



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

#10 attachment  
09/917789

(51) International Patent Classification: <b>A61K 38/00</b>	<b>A2</b>	(11) International Publication Number: <b>WO 00/21550</b> (43) International Publication Date: 20 April 2000 (20.04.2000)
(21) International Application Number: <b>PCT/US99/24221</b> (22) International Filing Date: <b>13 October 1999 (13.10.1999)</b> (30) Priority Data: 60/103,975 13 October 1998 (13.10.1998) US 60/136,631 27 May 1999 (27.05.1999) US (60) Parent Application or Grant PRESIDENT AND FELLOWS OF HARVARD COLLEGE [/]; (). TSAL, Li-Huei [/]; (). PATRICK, Gentry, N. [/]; (). LEE, Ming, Sum [/]; (). BROOK, David, E. ; ().	Publish d	
(54) Title: METHODS AND COMPOSITIONS FOR TREATING NEURODEGENERATIVE DISEASES (54) Titre: METHODES ET COMPOSITIONS DE TRAITEMENT DES MALADIES NEURODEGENERATIVES		
(57) Abstract <p>The present invention relates to methods of preventing or treating neurodegenerative diseases by administering an antagonist or inhibitor of p25. In particular, the invention relates to methods of preventing or treating a neurodegenerative disease by administering a calpain antagonist or inhibitor, or a cation antagonist or inhibitor, which reduces the truncation or conversion of p35 to p25.</p> <p>(57) Abrégé <p>La présente invention se rapporte à des méthodes de prévention ou de traitement des maladies neurodégénératives consistant à administrer un antagoniste ou un inhibiteur de p25. L'invention se rapporte notamment à des méthodes de prévention ou de traitement d'une maladie neurodégénérative, qui consistent à administrer un inhibiteur ou antagoniste de calpaïne, ou un inhibiteur ou antagoniste de cations, qui réduit la troncature ou la conversion de p35 en p25.</p></p>		

09/917789

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>A61K 38/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/21550</b> <b>(43) International Publication Date:</b> 20 April 2000 (20.04.00)
<b>(21) International Application Number:</b> PCT/US99/24221 <b>(22) International Filing Date:</b> 13 October 1999 (13.10.99)  <b>(30) Priority Data:</b> 60/103,975 13 October 1998 (13.10.98) US 60/136,631 27 May 1999 (27.05.99) US  <b>(71) Applicant:</b> PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; Holyoke Center, Suite 727, 1350 Massachusetts Avenue, Cambridge, MA 02138 (US).  <b>(72) Inventors:</b> TSAI, Li-Huei; 81 Farnham Street, Belmont, MA 02178 (US). PATRICK, Gentry, N.; 1 Woodman Street, No. 1, Jamaica Plain, MA 02130 (US). LEE, Ming, Sum  <b>(74) Agents:</b> BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).	<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	
<b>(54) Title:</b> METHODS AND COMPOSITIONS FOR TREATING NEURODEGENERATIVE DISEASES  <b>(57) Abstract</b>  The present invention relates to methods of preventing or treating neurodegenerative diseases by administering an antagonist or inhibitor of p25. In particular, the invention relates to methods of preventing or treating a neurodegenerative disease by administering a calpain antagonist or inhibitor, or a cation antagonist or inhibitor, which reduces the truncation or conversion of p35 to p25.		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**D scription**

5

10

15

20

25

30

35

40

45

50

55

-1-

## METHODS AND COMPOSITIONS FOR TREATING NEURODEGENERATIVE DISEASES

### BACKGROUND OF THE INVENTION

Mechanisms for many neurodegenerative diseases, including Alzheimer's Disease (AD), have evaded scientists. The cause of many neurodegenerative diseases is largely unknown, and hence treatment for these diseases are often lacking or ineffective.

AD is a degenerative disease characterized by progressive loss of neurons with a principle clinical manifestation of dementia. AD afflicts a very high proportion of the elderly with a lengthy progression. Although at least 5% to 10% of cases are familial, most cases are sporadic in incidence. The major pathological feature of AD includes the amyloid plaques that deposit extracellularly and cytoplasmic filamentous material that accumulates in the soma and in neurites. AD is associated with neuronal loss and pathological lesions in parts of the brain, including the hippocampus, amygdala and cerebral cortex.

A need exists to determine the mechanisms and causes of AD and other neurodegenerative diseases. A further needs exists to develop effective methods for the prevention, diagnosis and treatment of these diseases.

### SUMMARY OF THE INVENTION

The applicants have discovered the mechanisms which lead to neurodegenerative diseases (ND). The present invention takes advantage of the discovery that cleavage of p35, resulting in p25, triggers events that lead to NDs, such as Alzheimer's Disease. A protein, called p35, is cleaved, resulting in two proteins, p25 and p10. The applicants have also determined that this cleavage of p35 is performed by a protease, referred to as "calpain." Once p35 is cleaved by calpain, p25 accumulates in the brain and causes a series of events. p25 associates with another protein, cdk5, and makes cdk5 resistant to its normal regulation, and activates it. The p25/cdk5 complex or kinase induces phosphorylation of tau which

causes the formation of neurofibrillary tangles (NFT) and neuronal loss, symptoms associated with several NDs. This discovery has led to various forms of treatment for NDs that are encompassed by the present invention.

Accordingly, the invention relates to a method of preventing or treating a neurodegenerative disease in an individual, comprising administering to the individual an amount (e.g., an effective amount) of one or more compounds that reduce conversion of p35 to p25 in neurological tissue (e.g., brain or spinal tissue). The compound responsible for inhibiting or reducing the conversion of p35 to p25 is a compound that inhibits calpain or divalent cations including calcium. The method further includes administering both an inhibitor of the p35 to p25 conversion and p35 to the individual. A neurodegenerative disease is associated with neurofibrillary tangles and accumulation of p25 in the brain. The types of neurodegenerative diseases are dementias and neurodegenerative diseases associated with stroke, neurodegenerative diseases associated with myocardial infarction, neurodegenerative diseases associated with ischemia, and neurodegenerative diseases associated with other oxidative stresses. Examples of neurodegenerative disease are Alzheimer's disease, Corticobasal degeneration, Dementia pugilistica, Down's syndrome, Frontotemporal dementias and Parkinsonism linked to chromosome 17, Myotonic dystrophy, Niemann-Pick disease, Parkinson-dementia complex of Guam, Pick's disease, postencephalic Parkinsonism, prion disease with tangles, progressive supranuclear palsy, lower lateral sclerosis, Huntington's Disease, subacute sclerosing panencephalitis and multiple sclerosis.

The invention also pertains to methods of preventing or treating a neurodegenerative disease in an individual, comprising administering to the individual an amount of one or more of the following compounds: a compound that inhibits the activation of cdk5 kinase by p25, a compound that reduces phosphorylation of tau by a p25/cdk5 kinase, a compound that reduces accumulation of p25 in the brain, a calpain inhibitor or antagonist that reduces conversion of p35 to p25, or a cation inhibitor (e.g.,  $Mn^{2+}$ ,  $Sr^{2+}$ ,  $Ba^{2+}$ ,  $Mg^{2+}$  or  $Ca^{2+}$  inhibitors). Examples of the calpain inhibitor or antagonist are calpeptin, N-acetyl-leucyl-leucyl-methionine (ALLM), N-acetyl-leucyl-leucyl-norleucinal (ALLN), calpastatin, AK275, MDL28170, E64, leupeptin and calpain inhibitor I. Calcium inhibitors

5 include MKA01, omega-conotoxin and Sb201823-A.

10 The methods of the present invention relate to preventing or treating an individual having a neurodegenerative disease comprising administering an amount of one or more calpain inhibitors or antagonists and at least one other composition  
5 used for preventing or treating neurodegenerative disease. Compositions used for treating neurodegenerative diseases include, for example, COMT inhibitors, non-  
15 ergot DE dopamine agonists, monoamine oxidase inhibitors and ropinirole hydrochloride.

20 In addition to therapeutic methods, the present invention embodies methods of inhibiting or reducing conversion of p35 to p25 in neuronal tissue comprising contacting a calpain inhibitor or antagonist, and/or a cation inhibitor or antagonist with the neuronal tissue. The present invention also includes methods for preventing or reducing neurofibrillary tangles comprising contacting a calpain  
25 inhibitor or antagonist, and/or a cation inhibitor or antagonist with neuronal tissue. These methods can occur *in vivo* or *in vitro*.

30 Methods of diagnosing or aiding in the diagnosis of a neurodegenerative disease, or methods of determining the presence or absence of a neurodegenerative disease in an individual, are also encompassed by the present invention. These methods comprise determining the presence, absence and optionally, the level, of  
20 p25 in a sample obtained from the individual. If the level of p25 is determined, it can be compared to the level of p25 determined with a control or standard. The presence of p25 in the sample indicates the presence of a neurodegenerative disease, and the absence of p25 indicates the absence of a neurodegenerative disease. When  
35 the level of p25 is assessed, an increased level of p25 in the sample indicates that the ND or symptoms thereof has worsened, or that treatment is ineffective. A decreased level of p25 indicates the ND or symptoms thereof has gotten better, or that treatment is effective. In addition to assessing p25, p35 can also be assessed. An  
40 increased level of p25 and a decreased level of p35 relative to a standard indicates that the ND or symptoms thereof has gotten better or that treatment is effective, and  
25 a decreased level of p25 and an increased level of p35 indicates that the ND or symptoms thereof has worsened, or that treatment is ineffective. A ratio of p25 and  
30 p35 can be formed and compared to a standard.

5 The present invention also encompasses compounds for the prevention or  
treatment of a neurodegenerative disease, including a compound that inhibits the  
10 association of p25 with cdk5; a compound that inhibits the activation of cdk5 by  
p25; a compound that reduces the conversion of p35 to p25; a compound that  
5 reduces the phosphorylation of tau by p25/cdk5 kinase; a compound that inhibits  
calpain; a compound that inhibits a cation; and a compound that is an agonist of p35.  
15 The compound can be an antibody or antibody fragment that is specific to p25. The  
compound can also be a polypeptide or a nucleic acid construct encoding any one of  
these compounds.

20 The present invention offers effective treatments for neurodegenerative  
diseases. The methods of the present invention also provide assays that allow for  
efficient diagnosis of neurodegenerative diseases.

#### 25 BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-D show that calcium ions induce the proteolytic cleavage of p35  
15 and subsequent accumulation of p25. Figure 1A is a schematic diagram of the cdk5  
activator, p35, and shows proteolytic cleavage of p35 between residues 98 and 99,  
30 resulting in the neurotoxic 25kDa C-terminal fragment, p25. Figure 1B is a  
photograph of a western blot of p35 showing the effects of adding chloride salts of  
different cations as indicated to fresh mouse brain lysates. The first lane is the  
35 control which was COS cell lysate transfected with recombinant p25. Figure 1C is a  
photograph of a western blot using fresh mouse brain lysates treated with 1mM  
calcium chloride for the indicated time. 100µg of the lysates was run on a 11%  
40 polyacrylamide gel and probed with a polyclonal anti-p25 antibody. Figure 1D is a  
photograph of a western blot of mouse brain lysates treated with increasing  
25 concentrations of calcium chloride (0, 100 µM, 200 µM, 300 µM, 400 µM, 500  
µM, 600 µM, 700 µM, and 800 µM) and assessed for p25 accumulation with an  
45 anti-p25 antibody.

Figures 2A-F show that calcium-dependent protease calpain mediates p35  
cleavage. Figure 2A is a photograph of a western blot in which 1mM calcium  
50 chloride was added to mouse brain lysate to stimulate p35 cleavage in the presence  
of the following inhibitors: 2mM calpeptin (lane 3), 5µM calpain inhibitor II (lane



5  
10  
15  
20  
25  
30  
35  
40  
45  
50  
55

4), 1mM PMSF (lane 5), 1 $\mu$ g/ $\mu$ l aprotinin (lane 6), 1 $\mu$ g/ $\mu$ l pepstatin (lane 7), 1 $\mu$ g/ $\mu$ l leupeptin (lane 8), and 10 $\mu$ M roscovotine (lane 9). Figure 2B is a photograph of a western blot showing samples that were run on an 11% acrylamide gel and probed with an anti-p25 antibody (as done in Figure 2A), but samples were run on an 8% acrylamide gel and probed with an antibody against the endogenous calpain substrate non-erythroid  $\alpha$ -spectrin. Figure 2C is a photograph of a western blot, assayed for p35 conversion, showing fresh mouse brain lysates treated with 1mM calcium chloride and the indicated amount of the calpain inhibitors calpeptin or calpain inhibitor II. Figure 2D is a photograph of a western blot assays for p35 cleavage activity, showing fresh mouse brain lysates that were fractionated into 17 fractions by a 10-25% glycerol gradient. Figure 2E is a photograph of a western blot of the same fractions as in Figure 2D, but fractions were incubated with frozen and rethawed mouse brain lysate and assayed for spectrin-cleavage activity. Figure 2F is a photograph of a western blot showing a p35 immunoprecipitation from mouse brain lysates that was treated with purified recombinant m-calpain or  $\mu$ -calpain for the amounts indicated.

Figures 3A-F show that ionomycin and glutamate can stimulate conversion of p35 to p25 in primary cortical neurons. Figures 3A-3F are photographs of western blots. Figure 3A: Upper panel: day 7-cultured E17-E19-dissociated rat cortical neurons were treated with the indicated amount of ionomycin and assayed for p35 cleavage. Lower panel: the same set of lysates were assayed for cleavage of non-erythroid  $\alpha$ -spectrin. Figure 3B: Upper panel: 5 $\mu$ M ionomycin was added to cultures treated with increasing amount of the calpain-inhibitor calpeptin as indicated and assayed for p35 cleavage. Lower panel: samples were assayed for spectrin cleavage. Figure 3C shows cultures that were treated with 5 $\mu$ M ionomycin and harvested at 4 time points (0hr, 1hr, 2hr, and 3hr). Figure 3D: Upper panel: cultures were treated with 500 $\mu$ M glutamate and harvested every hour for 5 hours. Samples were assayed for p35 cleavage. Lower panel: samples were assayed for spectrin cleavage. Figure 3E: Upper panel: 500 $\mu$ M glutamate was added to cultures treated with different amounts of calpeptin (no inhibitor, 4  $\mu$ M, 40 $\mu$ M and 200 $\mu$ M). Samples were assayed for p35 cleavage activity. Lower panel: samples were

assayed for spectrin cleavage. Figure 3F shows cultures that were treated with increasing concentration of glutamate (0 $\mu$ M, 1 $\mu$ M, 10 $\mu$ M, 100 $\mu$ M, 1mM and 10mM) and assayed for p35 cleavage.

Figures 4A-E show that A $\beta$ (25-35) enhances H<sub>2</sub>O<sub>2</sub>-mediated conversion of p35 to p25. Figures 4A-E are photographs of western blots. Figure 4A: Upper panel: increasing amounts of H<sub>2</sub>O<sub>2</sub> were added to 14-day-old rat cortical neurons. Cells were lysed in ELB lysis buffer and assayed for p35 cleavage. Lower panel: samples were assayed for cleavage of spectrin. Figure 4B: Upper panel: 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> was added to 7-day-old neurons treated with increasing amounts of the calpain inhibitor calpeptin, and the samples were assayed for p35 cleavage. Lower panel: samples were assayed for cleavage of spectrin. Figure 4C: western blot of p35 in ipsilateral and contralateral cortices after 4 hours of permanent ischemia treatment. Figure 4D: Upper panel: 14-day old cortical neurons were treated with 100 $\mu$ M H<sub>2</sub>O<sub>2</sub> and/or 20 $\mu$ M A $\beta$ (25-35) as indicated. Neurons were then lysed and assayed for p35 cleavage. Lower panel: samples were assayed for cleavage of spectrin. Figure 4E is a schematic showing that A $\beta$ , oxidative stress and loss of calcium homeostasis lead to cleavage of p35 and accumulation of p25, which results in deregulation of cdk5 activity.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for preventing or treating neurodegenerative diseases (ND) by reducing the conversion or cleavage of a protein, referred to as "p35." When p35 is cleaved, it results in a protein called "p25." It has been discovered that cleaving p35 into p25 leads to the formation of neurofibrillary tangles (NFT) and neuronal loss. NFTs and neuronal loss are associated with several NDs. A protease, referred to as "calpain," causes the truncation of p35 to p25. The term "calpain" includes the protein in its natural form, including various isoforms, such as  $\mu$ -calpain and m-calpain; and the protein in a modified form.

Non-diseased brains have high levels of p35, and correspondingly low levels of p25, whereas Alzheimer's Disease (AD) brains have lower levels of p35 and

5 correspondingly high levels of p25. This is so because p35 has been truncated into  
p25. In non-diseased brains, a kinase, referred to as cdk5, functions normally.  
10 However, in AD brains, p25 deregulates cdk5, thereby making cdk5 more active  
than normal. p25 interacts with cdk5 and causes cdk5 to be overactive. This  
5 p25/cdk5 kinase increases phosphorylation of tau, relative to the amount of  
phosphorylation by a p35/cdk5 kinase. These events impair the integrity of the  
15 cytoskeleton, which ultimately results in morphological degeneration, and apoptosis  
or death of neurons; thereby leading to ND. These events are described in greater  
detail below and in the Exemplification section.

20 The present invention relates to methods for treating or preventing a ND in  
an individual by inhibiting or reducing the conversion of p35 to p25. Reduction of  
the conversion can be partial or complete. Inhibiting or reducing the conversion of  
p35 to p25 can be accomplished by a calpain inhibitor or antagonist. "Treating a  
25 ND" refers to alleviating or ameliorating one or more symptoms commonly  
associated with the ND. "Preventing a ND" refers to preventing one or more  
symptoms of the ND from occurring and/or from worsening. For example, some  
symptoms associated with AD are an increase in NFTs and an increase in neuronal  
30 loss, resulting in dementia. In one embodiment, treating AD refers to decreasing the  
amount of NFT and/or neuronal loss. Preventing AD refers to preventing the  
20 increase in NFTs and/or neuronal loss, or preventing these symptoms from  
worsening.

35 In addition to inhibiting the conversion of p35 to p25, the present invention  
takes advantage of the association of p25 with cdk5, once p25 is formed. The  
invention further relates to a method of preventing or treating a neurodegenerative  
40 25 disease in an individual by administering a compound that inhibits deregulation  
cdk5 kinase through inhibition of an association or interaction between p25 and  
cdk5. Reducing the interaction between p25 and cdk5 results in preventing or  
45 decreasing the phosphorylation of tau, and the formation of NFTs and neuronal  
degeneration.

30 Another embodiment of the invention relates to decreasing the  
phosphorylation of tau, through the p25/cdk5 pathway. Once p25 is formed and  
50 interacts with cdk5, the p25/cdk5 complex (e.g., p25/cdk5 kinase) increases

phosphorylation of tau, which eventually leads to the cause of ND. Hence, the present invention pertains to methods of preventing or treating a ND in an individual by administering a compound that reduces phosphorylation of tau with a p25/cdk5 kinase. Examples of such compounds include roscovitine and olomocine.

In addition to methods of preventing or treating ND, the present invention embodies methods for inhibiting or reducing conversion of p35 to p25, or reducing NFTs in neuronal tissue (e.g., brain tissue or spinal cord tissue) by contacting a calpain inhibitor or antagonist, and/or a cation inhibitor or antagonist with the neuronal tissue. This embodiment can be performed *in vivo* or *in vitro*.

A ND refers to a disease that impairs neurological or brain function through degeneration of neuronal tissue (e.g., spinal tissue or brain tissue). A ND also includes those diseases that are associated with neurofibrillary tangles and/or accumulation of p25 in the brain. Types of ND are dementias and neurodegenerative diseases associated with stroke, neurodegenerative diseases associated with myocardial infarction, neurodegenerative diseases associated with ischemia, and neurodegenerative diseases associated with other oxidative stresses. Examples of ND include, but are not limited to, Alzheimer's disease, Corticobasal degeneration, Dementia pugilistica, Down's syndrome, Frontotemporal dementias and Parkinsonism linked to chromosome 17, Myotonic dystrophy, Niemann-Pick disease, Parkinson-dementia complex of Guam, Pick's disease, postencephalic Parkinsonism, prion disease with tangles, progressive supranuclear palsy, lower lateral sclerosis, Huntington's Disease, subacute sclerosing panencephalitis and multiple sclerosis.

Compounds of the present invention refer to those compounds that reduce or inhibit the conversion of p35 to p25, including a calpain inhibitor or antagonist. An inhibitor or antagonist is a compound that reduces the function of a molecule (e.g., p25 or p25/cdk5 kinase), partially or completely. Antagonists or inhibitors include molecules which inhibit or decrease function or the biological activity of another molecule or protein. The biological activity of p25 refers to its ability to associate with cdk5, induce the phosphorylation of tau, cause the formation of NFTs and/or increase neuronal loss. The biological activity of the p25/cdk5 kinase refers to its ability to phosphorylate tau and induce the formation of NFTs and neuronal loss.

5 Antagonists include antibody or antibody fragments, peptide mimetics molecules,  
antisense molecules that hybridize to nucleic acid which encodes p25, ribozymes,  
10 aptimers, or small molecule inhibitors that are specific for p25 or the nucleic acid  
that encodes a p25 antagonist. A calpain antagonist is an antagonist that reduces  
5 conversion of p35 to p25, which, in turn, inhibits deregulation of cdk5 and/or  
reduces phosphorylation of tau. In addition to a calpain antagonist, compounds of  
15 the present invention include compounds that inhibit the association of p25 with  
cdk5, compounds that inhibit the deregulation of cdk5 by p25, and compounds that  
reduce the phosphorylation of tau by p25/cdk5 kinase. Such antagonists inhibit  
20 and/or reduce the formation of NFT and/or neuronal loss. Examples of calpain  
inhibitors or antagonists are calpeptin, N-acetyl-leucyl-leucyl-methionine (ALLM),  
N-acetyl-leucyl-leucyl-norleucine (ALLN), calpastatin, AK275, MDL28170, E64,  
leupeptin and calpain inhibitor I.

25 Calpain is the protease that is responsible for conversion of p35 to p25. The  
15 data described herein show that a calpain antagonist prevents conversion of p35 to  
p25, and prevents the formation of NFTs. Calpain is dependent on cations, and in  
particular, divalent cations (e.g.,  $Mn^{2+}$ ,  $Sr^{2+}$ ,  $Ba^{2+}$ ,  $Mg^{2+}$  and  $Ca^{2+}$ ). Calpain is  
30 dependent on calcium, and less so on other divalent cations. Therefore, reducing  
interaction of calcium and/or other divalent cations with calpain impedes or inhibits  
20 calpain. The experiments described herein illustrate that both calpain antagonists as  
well as cation antagonists reduce the formation of p25 and NFT. The present  
35 invention embodies treating or preventing a ND by administering a cation inhibitor  
or antagonist, such as a calcium and/or magnesium inhibitor or antagonist. A few  
examples of calcium inhibitors are MKA01, omega-conotoxin and SB201823-A.  
40 The present invention utilizes known cation antagonists and known calpain  
antagonists, or those that are developed or discovered in the future.

45 One type of antagonist is an antibody. Antibodies specific to calpain, p25, or  
the p25/cdk5 kinase can be raised against an appropriate immunogen, such as  
isolated and/or recombinant proteins or portion thereof (including synthetic  
30 molecules, such as synthetic peptides). One can also raise antibodies against a host  
cell which expresses a recombinant antigen (e.g., calpain, p25, or the p25/cdk5  
50 kinase). Additionally, cells expressing a recombinant antigen, such as transfected

cells, can be used as immunogens or in a screen for antibody which binds receptor.

Techniques known in the art can be employed to prepare an immunizing antigen and to produce polyclonal or monoclonal antibodies. The art contains a variety of these methods (see e.g., Kohler *et al.*, *Nature*, 256: 495-497 (1975) and Eur. J. Immunol. 6: 511-519 (1976); Milstein *et al.*, *Nature* 266: 550-552 (1977); Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, fusing a suitable immortal or myeloma cell line, such as SP2/0, with antibody producing cells can produce a hybridoma. Animals immunized with the antigen of interest and, preferably, an adjuvant provide the antibody producing cell (cells from the spleen or lymph nodes). Selective culture conditions isolate antibody producing hybridoma cells while limiting dilution techniques produce them. One can use suitable assays such as ELISA to select antibody producing cells with the desired specificity.

Other suitable methods can be employed to produce or isolate antibodies of the requisite specificity. Examples of other methods include selecting recombinant antibody from a library or relying upon immunization of transgenic animals such as mice which are capable of producing a full repertoire of human antibodies (see e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 2551-2555 (1993); Jakobovits *et al.*, *Nature*, 362: 255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807).

In addition to use of antagonists, the present invention also includes use of agonists of p35. An agonist of p35 is one that enhances or promotes the function or biological activity of p35. The biological activity of p35 refers to the ability of p35 to prevent the occurrence of NFT, and other symptoms of ND. In addition to administering a calpain inhibitor or antagonist that inhibits conversion of p35 to p25, the present invention also includes administering (e.g., prior to, simultaneously, or after the calpain inhibitor or antagonist) p35 or a portion thereof that retains the biological activity of p35 (e.g., a modified form of p35). Routes and times of administration are further described below.

5 The present invention embodies administering a calpain or cation antagonist,  
along with at least one other composition (e.g., a drug) used for preventing or  
10 treating neurodegenerative disease. Drugs used for treating neurodegenerative  
disease are, for example, COMT inhibitors, non-ergot DE dopamine agonists,  
5 monoamine oxidase inhibitors and ropinirole hydrochloride. Drugs that are known  
or developed in the future can be administered with the calpain or cation antagonist.

15 One antagonist can be co-administered with at least one other compound  
including another antagonist, an agonist or a drug used for treating ND. The co-  
administration of these compounds can occur simultaneously or sequentially in time.

20 The compound used for treating ND, a second antagonist or an agonist can be  
administered before, after or at the same time as the antagonist. Thus, the term "co-  
administration" is used herein to mean that the antagonist and any additional  
compounds will be administered at times to achieve reduction or prevention of at  
25 least one of the following: conversion from p35 to p25, association of p25 with  
15 cdk5, phosphorylation of tau by the p25/cdk5 kinase, formation of NFT, or neuronal  
loss. The methods of the present invention are not limited to the sequence in which  
these compounds are administered, so long as the additional compound is  
30 administered close enough in time to produce the desired effect.

#### *Immunological Assessment of Neurodegenerative Diseases*

35 20 The present invention also includes methods for diagnosing or aiding in the  
diagnosis of a ND. To do so, the presence, absence or level of p25 is assessed. In  
addition to p25, other markers for ND can be assessed including p25/cdk5 and p35.  
40 A sample from the individual being tested is obtained. The sample can be any  
bodily material that contains p25, or metabolites of the p35/p25 conversion.  
25 Examples of samples include: cerebral spinal fluid, lymph, blood, sputum, tissue  
(e.g., from the brain or spinal cord), urine, saliva, plasma, mucus, or other cell  
45 samples that contain p25. The sample is preferably brain tissue, tissue from the  
spinal cord, or cerebral spinal fluid.

Once a sample is obtained, the presence or absence of p25 is assessed or  
50 30 measured. The presence of p25 in a sample indicates that the individual has a ND,  
50 whereas a decrease of p25 in a sample indicates that the individual does not have a

5 ND. The level of p25 can also be assessed. A level of p25, as compared to standard  
or control, indicates that the individual has a ND, or that the ND has worsened e.g.,  
10 due to ineffective treatment. Conversely, a decreased level of p25, as compared to a  
control, indicates the absence of a ND, or that ND has gotten better, e.g., that  
5 treatment has been effective. Preferably, both p35 and p25 are assessed. Increased  
levels of p35 indicate the absence of a ND, or the effective treatment of ND; while  
15 decreased levels of p35 indicates the presence of ND, or the worsening of ND,  
relative to a control or standard. A p25/p35 ratio can be calculated and used as a  
measured of the presence or absence of a ND.

20 Several suitable assays to measure the presence of p25, p35 or the p25/cdk5  
kinase, referred to collectively as "ND markers." Suitable assays encompass  
immunological methods, such as radioimmunoassay, flow cytometry, enzyme-linked  
25 immunosorbent assays (ELISA), and chemiluminescence assays. Any method  
known or developed later can be used for measuring p25, p35 or the p25/cdk5  
15 kinase.

The assays utilize antibodies reactive with ND markers, portions thereof or  
30 functional fragments thereof. The terms polyclonal and monoclonal refer to the  
degree of homogeneity of an antibody preparation, and are not intended to be limited  
to particular methods of production. Methods of making antibodies are known in  
20 the art, and are described herein.

35 In several of the embodiments, immunological techniques detect ND marker  
levels by means of an anti-ND marker antibody (i.e., one or more antibodies). The  
term "anti-ND marker antibody" includes monoclonal antibodies polyclonal  
antibodies, and/or mixtures thereof. For example, these immunological techniques  
40 can utilize mixtures or a cocktail of polyclonal and/or monoclonal antibodies.  
25

Immunological assays or techniques can be employed to determine the  
presence, absence or level of a ND marker in a biological sample. One or more ND  
45 markers can be measured in a sample. In determining the amounts of a ND marker,  
an assay generally includes combining the sample to be tested with an antibody  
30 having specificity for the ND marker, under conditions suitable for formation of a  
complex between antibody and the ND marker, and detecting or measuring (directly  
50 or indirectly) the formation of a complex.



5  
10  
15  
20  
25  
30  
35  
40  
45  
50  
55

Methods of combining sample and antibody, and methods of detecting complex formation are selected to be compatible with the assay format. Suitable labels can be detected directly, such as radioactive, fluorescent or chemiluminescent labels. They can also be indirectly detected using labels such as enzyme labels and other antigenic or specific binding partners like biotin. Examples of such labels include fluorescent labels such as fluorescein, rhodamine, CY5, APC, chemiluminescent labels such as luciferase, radioisotope labels such as  $^{32}\text{P}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , enzyme labels such as horseradish peroxidase, and alkaline phosphatase,  $\beta$ -galactosidase, biotin, avidin, spin labels and the like. The detection of antibodies in a complex can also be done immunologically with a second antibody which is then detected.

#### Radioimmunoassay:

25  
30  
35  
40  
45  
50  
55

A radioimmunoassay can be employed to measure the ND markers. A ND marker can be assessed by a radioimmunoassay by first obtaining a suitable sample to be tested. The sample is contacted with an anti-ND marker antibody (e.g., an anti-ND marker antibody comprising a radioactive label, or an anti-ND marker antibody comprising a binding site for a second antibody that has a radioactive label) preferably in an amount in excess of that required to bind the ND marker present, and under conditions suitable for the formation of labeled complexes between the anti-ND marker antibody. The formation of the complex in the samples is determined by detecting or measuring the radioactivity in the sample.

#### Enzyme-Linked Immunosorbent Assays (ELISA):

40  
45  
50  
55

Detection of a ND marker in a suitable sample can also occur by employing ELISA methods. To determine a measurement of a ND marker using an ELISA assay in a suitable sample, one contacts the sample with an anti-ND marker antibody, and then measures the formation of a complex between the anti-ND marker antibody and the ND marker in the sample. The ND marker can be measured by direct, indirect, sandwich or competitive ELISA formats. An antibody can be conjugated with labels such as biotin and HRP-streptavidin.

5

10

A solid support, such as a microtiter plate, dipstick, bead, or other suitable support, can be coated directly or indirectly with an anti-ND marker antibody. For example, an anti-ND marker antibody can coat a microtiter well, or a biotinylated anti-ND marker Mab can be added to a streptavidin coated support. A variety of

5 immobilizing or coating methods as well as a number of solid supports can be used, and can be selected according to the desired format.

15

20

In one embodiment, the sample or ND marker standard is combined with the solid support simultaneously with the detector antibody. Optionally, this composition can be combined with a one or more reagents by which detection is

10 monitored. For example, the sample can be combined with the solid support simultaneously with (a) HRP-conjugated anti-ND marker Mab, or (b) a biotinylated anti-ND marker Mab and HRP-streptavidin.

25

A known amount of the ND marker standard can be prepared and processed as described above for a suitable sample. This ND marker standard assists in

15 quantifying the amount of ND marker detected by comparing the level of ND marker in the sample relative to that in the standard.

30

35

40

45

A physician, technician, apparatus or a qualified person can compare the amount of detected complex with a suitable control to determine if the levels are increased or decreased. A variety of methods can determine the amount of an ND

20 marker in complexes. For example, when HRP is used as a label, a suitable substrate such as OPD can be added to produce color intensity directly proportional to the bound anti-ND marker Mab (assessed e.g., by optical density), and therefore to the ND marker in the sample. One can compare the results to a suitable control such as a standard, levels of ND marker in non-diseased individuals, and baseline

25 levels of ND marker in a sample from the same donor. For example, the assay can be performed using a known amount of a ND marker standard in lieu of a sample, and a standard curve established. One can relatively compare known amounts of the a ND marker standard to the amount of complex formed or detected.

50

A control or standard refers to the level of p25 and/or p35 in one or more

30 individuals who do not have the ND being tested. A positive control is a level of p25 and/or p35 in one or more individuals who have the ND being tested. A sample to be tested from an individual who does not have the ND has a level of p25 and/or

55

5 p35 closer to that of the levels from individuals who do not have the ND, and farther  
away from the levels of the positive control. A sample from an individual who does  
10 have the ND has a level of p25 and/or p35 closer to the levels of the positive control,  
and farther from the levels from samples of individuals who do not have the ND.

5 ND markers can be assessed using methods in the art or methods later  
developed in the future.

15 The invention also includes methods for determining whether an individual  
is likely (e.g., whether it is probable for an individual) to contract a ND by  
determining whether a polymorphism of a gene that encodes p35 exists. Persons  
20 who have a higher probability of getting a ND are persons who are likely to have a  
gene that encodes a form of p35 that is more susceptible to cleavage.

25 *Administration and dosages:*

The terms "pharmaceutically acceptable carrier" or a "carrier" refer to any  
generally acceptable excipient or drug delivery composition that is relatively inert  
30 and non-toxic. The antagonist or agonist can be administered with or without a  
carrier. Exemplary carriers include calcium carbonate, sucrose, dextrose, mannose,  
albumin, starch, cellulose, silica gel, polyethylene glycol (PEG), dried skim milk,  
rice flour, magnesium stearate, and the like. Suitable formulations and additional  
35 carriers are described in Remington's Pharmaceutical Sciences, (17th Ed., Mack  
20 Pub. Co., Easton, PA).

Suitable carriers (e.g., pharmaceutical carriers) also include, but are not  
40 limited to sterile water, salt solutions (such as Ringer's solution), alcohols,  
polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch,  
magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid esters,  
25 hydroxymethylcellulose, polyvinyl pyrrolidone, etc. Such preparations can be  
sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives,  
45 stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure,  
buffers, coloring, and/or aromatic substances and the like which do not deleteriously  
react with the active compounds. They can also be combined where desired with  
50 other active substances, e.g., enzyme inhibitors, to reduce metabolic degradation. A

5 carrier (e.g., a pharmaceutically acceptable carrier) is preferred, but not necessary to administer an antagonist or agonist.

10 For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or  
5 implants, including suppositories. In particular, carriers for parenteral administration include aqueous solutions of dextrose, saline, pure water, ethanol,  
15 glycerol, propylene glycol, peanut oil, sesame oil, polyoxyethylene-polyoxypropylene block polymers, and the like. Ampules are convenient unit dosages.

20 The antagonist, inhibitor or agonist is administered to neuronal tissue affected by the disease, or the vasculature around or leading to the site. The antagonist or agonist can be administered systemically or locally. The antagonist or  
25 antagonist can be administer directly to the neuronal tissue by injection. If administered systemically, then the antagonist or agonist can target the neuronal  
15 tissue (e.g., the brain) using methods known in the art. The antagonist, inhibitor or agonist of the present invention can also be administered intravenously, parenterally,  
orally, nasally, by inhalation, by implant, by injection, or by suppository. The  
30 composition can be administered in a single dose or in more than one dose over a period of time to confer the desired effect.

20 Effective amounts of antagonist or agonist can vary according to the specific drug being utilized, the particular composition formulated, the mode of  
35 administration, the age, weight and condition of the patient, or whether the ND is being treated or prevented, for example. As used herein, an effective amount of the antagonist or agonist is an amount of the drug which reduces or prevents conversion  
40 of p35 to p25, interaction of p25 with cdk5, the phosphorylation of tau, or the formation of NFT. Dosages for a particular patient can be determined by one of  
25 ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

30 The following examples are meant to be illustrative and not limiting in any way.

## EXEMPLIFICATION:

## Example 1: Methods

## Chemicals and Antibodies

p25 antibody was raised against the whole protein and purified against GST-p25. Non-erythroid  $\alpha$ -spectrin antibody was purchased from Chemicon. PMSF, Pepstatin A, aprotinin, leupeptin, glutamate and all the metal chlorides were purchased from Sigma. Recombinant calpain I, recombinant calpain II, calpeptin, calpain inhibitor II and ionomycin were purchased from Calbiochem.  $H_2O_2$  was purchased from Fisher Scientific.  $A\beta(25-35)$  was purchased from Bachem.

## Primary cortical neuronal cultures

E17-E19 pregnant rats of the Long Evans strain were purchased from Harland Sprague-Dawley. Embryos were surgically removed and their cortices were dissected and cultured as described in Behl, C., *et al.*, *Cell* **77**, 817-27 (1994).. Cortical cultures were grown in basal growth media on 6-well plates coated with laminin and poly-D-lysine.

## Western blot analysis

Primary cortical cultures and whole mouse brains were lysed in ELB buffer (50mM Tris pH 7, 0.1% NP-40, 250mM NaCl, 5mM EDTA). Lysates was run on either 8% or 11% acrylamide gels, transferred to Immobilon-P membranes (Millipore), and probed with antibodies raised against p35 (1:1500) or spectrin (1:5000).

## Glycerol gradient

A 11ml glycerol gradient of 10% to 25% was made in ELB buffer. 300 $\mu$ l of fresh mouse brain lysates was layered on top of the gradient and spun at 40K rpm for 26 hours. Seventeen 600 $\mu$ l fractions were collected and analyzed as described below.

## Ischemia

Adult mice (C57BL/6), weighing 16-20 g, were anesthetized initially with

5 1.5% isoflurane and thereafter maintained in 1.0% isoflurane in 70% N<sub>2</sub>O and 30%  
10 O<sub>2</sub>. Ischemia was produced by inserting an 8.0 nylon monofilament suture coated  
with a silicone/hardener mixture (Heraeus Kulzer, Inc. South Bend, IN) into the  
right common carotid artery. The suture was advanced 9-10mm from the insertion  
5 site through the internal carotid artery, occluding the middle cerebral artery (MCA).  
Mice all woke up hemiplegic and were sacrificed with isoflurane 4 hours after the  
15 induction of ischemia.

#### Example 2: Results

20 Cyclin-dependent kinase 5 and its neuronal-specific activator p35 are  
10 required for neurite outgrowth and cortical lamination. p25, a proteolytic cleavage  
product of p35, is accumulated in brains of patients with Alzheimer's disease. The  
accumulation of p25 plays a role in the pathogenesis of Alzheimer's disease because  
25 conversion of p35 to p25 leads to deregulation of the cdk5 kinase which  
subsequently results in tau hyperphosphorylation and neuronal death. The p35/p25  
15 conversion is mediated by the calcium-dependent protease calpain. *In vitro*, addition  
of millimolar range of calcium ion to mouse brain lysate stimulates the conversion  
30 of p35 to p25, and inhibitors of calpain can completely inhibit the  
calcium-stimulated p35/p25 conversion. Using recombinant proteins, purified  
calpain can directly cleave p35 to produce p25. In primary cortical neuronal  
35 20 cultures, activation of calpain by ionomycin or glutamate causes a complete  
conversion of p35 to p25. Hydrogen peroxide can partially stimulate the p35/p25  
conversion while an amyloidogenic peptide A $\beta$ (25-35) renders neurons more  
40 susceptible to the hydrogen peroxide-induced conversion. In a mouse forebrain  
ischemia model, the activation of calpain tightly correlates with the conversion of  
25 p35 to p25. The calcium-dependent protease calpain cleaves p35 to p25 and thus  
provides a mechanism by which altered calcium homeostasis can change the  
45 properties of the tau-phosphorylating kinase cdk5.

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase that shows  
high homology to the human cell cycle regulatory kinase cdc2. Cdk5 kinase activity  
50 30 is only detected in brain lysates, where Cdk5 protein level is highest. p35, a  
neuronal-specific activator of cdk5, is highly expressed in post-mitotic neurons in

the central nervous system (CNS). During neuronal differentiation, the p35/cdk5 kinase is required for neurite outgrowth. p35 knockout mice displays severe cortical lamination defects and suffer from sporadic adult lethality and seizures. cdk5 knockout mice are perinatal lethal and display severe defects in the cortex, cerebellum, and the hippocampus. Thus, the p35/cdk5 kinase plays a pivotal role in CNS development.

Interestingly, p25, a truncated fragment of p35, was found to accumulate in brains of patients with Alzheimer's disease (AD) but not in age-matched control brains. The p25/cdk5 kinase, which was biochemically purified as a brain-specific histone H1 kinase, hyperphosphorylates tau *in vitro* and *in vivo*. In cortical neurons, introduction of p25/cdk5 leads to collapse of the cytoskeleton and apoptotic death. Therefore, understanding the mechanism that leads to p25 production sheds light on the pathogenesis of Alzheimer's disease. As the open-reading frame of p35 does not contain any introns, p25 can only be produced by proteolytic cleavage.

In an effort to elucidate the molecular events leading to conversion of p35 to p25, recapitulate of the proteolytic cleavage event *in vitro* was sought. Under normal lysis condition, p25 is nearly undetectable in brain lysates even after several days at 4°C. Various ions were initially added to test whether any of these facilitates p35 cleavage.  $Mn^{2+}$ ,  $Sr^{2+}$ ,  $Ba^{2+}$  and  $Ca^{2+}$  can induce the conversion of p35 to p25 at a concentration of 5mM. At a lower concentration of 1mM, only  $Ca^{2+}$  can induce cleavage of p35 (Fig. 1b). The p35/p25 conversion is most efficiently induced in fresh brain lysates. The efficiency of cleavage is much lower in frozen and rethawed brain lysates, indicating that the p35-cleaving activity is sensitive to freeze-thawing.

Since calcium is most effective in stimulating p35 conversion, the time necessary and concentration of calcium required for conversion to occur was determined. One minute after 1mM  $Ca^{2+}$  treatment, p25 is already visible. However, it took about 45 minutes for half, and 2 hour for the entire p35 pool to be cleaved to p25 (Fig. 1c). A titration of the amount of calcium shows that 200μM  $Ca^{2+}$  is the lowest concentration that can effectively stimulate the conversion of p35 to p25 (Fig. 1d). These results indicate that the existence of a protease in mouse brain lysates that can cleave p35 to produce p25, and that this protease is either itself sensitive to the concentration of calcium ion, or can be activated by other factors that

are sensitive to calcium ion.

p35 does not contain any consensus sequences for cleavage by known proteases. To identify the protease activated by calcium, protease inhibitors with diverse specificities for their effectiveness in inhibiting calcium-stimulated p35 conversion were tested. The aspartic protease inhibitor pepstatin, the serine protease inhibitors aprotinin and PMSF, and the cdk5 inhibitor roscovitine are all ineffective in inhibiting the calcium-stimulated proteolytic cleavage of p35 (Fig. 2a, lanes 5-7 and 9). The inability of roscovitine to affect cleavage indicates that cdk5 activity is not necessary for cleavage to occur. On the other hand, calpeptin and calpain inhibitor I, which inhibit the calcium-dependent cysteine protease calpain, completely inhibit p35 cleavage (lanes 3-4), while the cysteine protease inhibitor leupeptin partially inhibits p35 cleavage (lane 8). A titration of the two calpain-specific inhibitors shows that 0.4 $\mu$ M calpeptin and 0.5 $\mu$ M calpain inhibitor I are effective in inhibiting p35 conversion (Fig. 2c), consistent with the reported IC50 values for these inhibitors.

m-calpain (also known as calpain I) and  $\mu$ -calpain (also known as calpain II) are the two major isoforms of calpain found in the brain. The two calpains differ in their calcium requirements but have similar substrate specificities.  $\mu$ -calpain requires 3-50 $\mu$ M calcium for half-maximal activity, while m-calpain requires 0.2-1mM calcium for activity. Other divalent cations like Mn<sup>2+</sup>, Ba<sup>2+</sup> and Sr<sup>2+</sup> have also been shown to stimulate calpain activity at a high concentration. Both the ion stimulation profile and the inhibitor study point to the involvement of calpain in the proteolytic cleavage of p35.

To determine if calpain is activated by the conditions tested above *in vitro*, the cleavage of a well-characterized calpain substrate, non-erythroid  $\alpha$ -spectrin (also known as  $\alpha$ -fodrin) was examined. Both  $\mu$ - and m-calpain cleave  $\alpha$ -spectrin at two sites in a sequential manner. The first site is highly susceptible and is cleaved rapidly once calpain is activated, leading to an  $\alpha$ -spectrin breakdown product of 150kDa. Prolonged action of calpain leads to further cleavage to produce a 145kDa fragment. Appearance of the spectrin breakdown products tightly correlates with conversion of p35 to p25 (Fig. 2b). 1mM calcium, which stimulates conversion of



5 p35 to p25 in mouse brain lysates, also leads to cleavage of endogenous spectrin into  
the 145kDa and 150kDa fragments. Furthermore, spectrin cleavage is inhibited by  
10 addition of calpeptin, calpain inhibitor I, and leupeptin, showing that calpain  
activation tightly correlates with p35 cleavage.

5 Mouse brain lysates were fractionated by glycerol gradient centrifugation.  
These fractions were incubated with purified p35 to identify fractions containing p35  
15 cleavage activity (Fig. 2d). These fractions were also incubated with spectrin to  
identify the fractions containing calpain activity (Fig. 2e). Both p35 cleavage  
activity and spectrin cleavage activity are present in fractions 8 to 10, indicating that  
20 calpain activity cofractionates with p35 cleavage activity. These results  
cumulatively indicate that p35/p25 conversion lies downstream of calpain activation.

To determine if calpain directly cleaves p35, purified calpain was incubated  
25 with p35 purified by immunoprecipitation with a p35-specific antibody. Both  
purified m-calpain and  $\mu$ -calpain cleave p35 to produce p25 (Fig. 2f). Similar  
15 results were obtained by incubating purified calpains with  $^{35}\text{S}$ -labelled *in*  
*vitro*-translated p35. Therefore, calpain can directly cleave p35 to produce p25,  
30 indicating that it is the protease in mouse brain lysates that converts p35 to p25. In  
summary, it has been shown that *in vitro*, activation of calpain by calcium  
stimulation is necessary and sufficient for p35/p25 conversion.

20 Conditions for *in vivo* p35/p25 conversion were tested in rat primary cortical  
neuronal cultures. Since an elevation of calcium levels stimulates the conversion  
event *in vitro*, neurons were treated with the calcium ionophore ionomycin. In  
35 cortical neurons, 4 $\mu\text{M}$  ionomycin induces the cleavage of one-half of the  
endogenous p35 in 5 hours (Fig. 3a). When neurons are treated with 6 $\mu\text{M}$   
40 ionomycin, the entire pool of endogenous p35 is converted to p25. A time course  
analysis shows that most of the endogenous p35 is converted to p25 3 hours after  
5 $\mu\text{M}$  ionomycin treatment (Fig. 3c). Calpain activation was monitored by the  
45 breakdown of its endogenous substrate spectrin. The 280kDa spectrin is cleaved  
into the 150kDa and 145kDa fragments when ionomycin is added, indicating that  
30 calpain is activated by ionomycin (Fig. 3a). When the cell-permeable calpain  
inhibitor calpeptin was added to ionomycin-treated neurons, conversion of p35 to  
50 p25 is completely inhibited (Fig. 3b). Thus, in cortical neurons, calcium influx by

ionomycin treatment leads to calpain-dependent conversion of p35 to p25.

Excitatory amino acids, such as glutamate, have also been shown to cause a loss of calcium homeostasis in neurons which subsequently leads to a sustained elevation of intracellular calcium level. When increasing concentrations of glutamate was added to primary neuron cultures, it was found that a high concentration of glutamate can effectively induce the conversion of p35 to p25 (Fig. 3f). When neurons are treated with 500 $\mu$ M glutamate, most of the endogenous p35 is converted to p25 in 2 hours (Fig. 3d). Calpain-activation is responsible for p35 cleavage in glutamate-treated neurons, since the endogenous calpain substrate spectrin is cleaved into the characteristic 145kDa and 150kDa fragments. Furthermore, the calpain inhibitor calpeptin effectively inhibits proteolytic cleavage of p35 and spectrin (Fig. 3e).

Since there is increasing evidence that in Alzheimer's disease, the effects of A $\beta$ -initiated inflammatory and neurotoxic processes include excessive generation of free radicals and peroxidative injury in neurons, determining whether hydrogen peroxide can mediate conversion of p35 to p25 was studied. When primary cortical neurons was treated with hydrogen peroxide, it was found that 100 $\mu$ M to 1mM hydrogen peroxide can stimulate conversion of p35 to p25 (Fig. 4a). The spectrin cleavage pattern confirms that calpain is activated when the concentration of hydrogen peroxide is between 100 $\mu$ M to 1mM. The calpain inhibitor calpeptin efficiently inhibits cleavage of p35 as well as spectrin (Fig. 4b), indicating that hydrogen peroxide can also induce cleavage of p35 by activating the protease calpain. In addition, oxidative stress *in vivo* can induce p35/p25 conversion. In a forebrain ischemia model, 4 hours of ischemia produces an accumulation of p25 in the ipsilateral cortex but not in the contralateral cortex (Fig. 4c).

Since p25 was found to accumulate in brains of Alzheimer's patients but not in non-diseased brains, determining whether the amyloidogenic peptide A $\beta$ (25-35) can induce conversion of p35 to p25 was also studied. The A $\beta$ (25-35) peptide is a synthetic peptide corresponding to amino acid residues 25-35 of the Amyloid- $\beta$  protein, which is the primary constituent of senile plaques found in Alzheimer's brains. The A $\beta$ (25-35) peptide has been shown to aggregate and cause neuronal

5 death. A $\beta$ (25-35) itself does not induce conversion in neurons even after prolonged  
incubation (Fig. 4d). However, it renders neurons more susceptible to hydrogen  
10 peroxide-mediated conversion. When 100 $\mu$ M of hydrogen peroxide is added to  
neurons, only ~50% of endogenous p35 is converted to p25, but when 100 $\mu$ M of  
5 hydrogen peroxide is added to cortical neurons incubated with 20 $\mu$ M of A $\beta$ (25-35),  
over 80% of the endogenous p35 is converted to p25. Therefore, A $\beta$ (25-35)  
15 sensitizes neurons to the production of p25 caused by oxidative stress.

In summary, *in vitro* and *in vivo* evidence that the calcium-dependent  
protease calpain cleaves p35 to produce p25 has been provided. While p35 is a  
20 tightly regulated protein which allows cdk5 to be activated in a temporally- and  
spatially-specific manner, p25 causes cdk5 to be mislocalized and constitutively  
active. Thus, by cleaving p35, calpain does not downregulate cdk5 activity but  
25 rather, it completely alters the properties of cdk5 so that substrates such as tau and  
neurofilaments which are poorly phosphorylated by p35/cdk5 are now  
15 hyperphosphorylated by p25/cdk5. As such, while p35/cdk5 activity is necessary for  
proper CNS development and other functions of the mature nervous system,  
p25/cdk5 causes collapse of the cytoskeleton and apoptotic cell death.  
30

Increased calpain activity and altered calcium homeostasis are both observed  
in brains of patients with Alzheimer's disease. In particular, loss of calcium  
20 homeostasis has been implicated in causing tau hyperphosphorylation and neuronal  
apoptosis. The synergy between oxidative stress and the amyloidogenic A $\beta$ (25-35)  
35 peptide in causing p35/p25 conversion indicates that in some cases of Alzheimer's  
disease, calpain activation and cdk5 deregulation lies downstream of A $\beta$  (Fig. 4e).  
Given the potentially deleterious role cdk5 can play in Alzheimer's disease, the  
40 calpain-mediated p35 cleavage pathway serves as one of the targets for  
25 pharmacological intervention.

### 45 Example 3: Additional Experimental Procedures Supporting the role of p25

#### Chemicals and Antibodies

50 Cycloheximide (20 mg/mL stock) was purchased from Sigma and used at a

5

10

15

20

25

30

35

40

45

50

55

5 final concentration of 30  $\mu\text{g}/\text{mL}$  in  $t_{1/2}$  experiments. Hoechst dye was also  
purchased from Sigma. The following antibodies were used: p35: pAb neu-  
10 cyc(purified either with GST-p10 (N-terminal – GST-p10 purified) or GST-p25 (C-  
terminal – GST-p25 purified), 4E3 raised against whole protein, N-20 and C19  
5 (Santa Cruz); cdk5: mAb DC17<sup>41</sup>, pAb C8 (Santa Cruz); HA (mAb 12CA5); mAb  
6xHIS (Boehringer Mannheim); pAb  $\beta\text{gal}$  antibodies (Promega); pAb actin; mAb  
15 alpha and beta-tubulin (Sigma); Tau: dephosphorylated tau epitopes at Ser-199 and  
Ser-202- mAb Tau-1, (Boehringer Mannheim); phosphorylated epitopes at Ser-396  
and Ser-404 - mAb PHF-1, a gift from P. Davies; phosphorylated epitopes at N-  
20 terminal residues 2 - 10 - mAb Alz-50, a gift from P. Davies; AT8 was purchased  
from Innogenetics. Caspase-3 inhibitor Ac-DEVD-CHO was purchased from  
Sigma.

#### 25 Constructs and Viruses

Construction of CMV expression vectors for p10, p35, cdk5 and DNK5 was  
15 previously described. CMV-p25 (amino acids 98-307) was made by PCR using a 3'  
p35 primer and 5' deletion primer. CMV-p10 (amino acids 1-97) was made by PCR  
30 using a 5' p35 primer and 3' deletion primer. The p10 fragment was then cloned  
upstream of HA tag. p35 Gly2Ala was made by site directed mutagenesis using the  
following oligo: 5'-CAGACACCATGGCCACGGTG – 3' (SEQ ID NO: 1)  
35 Mutagenesis was verified by sequencing. Recombinant Herpes Simplex Virus  
20 constructs (a gift from Rachel Neve) were made according to a system previously  
described. The following viruses were used: HSV-  $\beta\text{gal}$ , HSV-p35, HSV-p25, and  
HSV-GFP-cdk5, and HSV-GFP-DNK5. Multiplicity of infection ranged from 0.1 to  
40 1 for all viruses used. HA-GSK3- $\beta$  mammalian expression construct (a gift from X.  
25 He); HIS6-htau40 mammalian expression construct (a gift from David Auprin,  
Pfizer)

#### 45 Tissues and Cell Culture

Brain tissues of 8 AD cases, 4 age-matched non-neurological cases, and one  
50 HD case were used in this study. Brain lysates were made from either Brodmann  
30 Areas 11, 21, or 45 by dounce homogenizing tissue in lysis buffer (50 mM Tris pH

5 7.5, 150 mM NaCl, 1% Triton-X, 10% glycerol, 5 mM EDTA, 1mM EGTA, 1mM  
10 DTT) plus inhibitors ( 2 µg/mL of aprotinin, 2 µg/mL of leupeptin, 1 µg/mL of  
pepstatin, 50 mM beta-glycerophosphate, 5 mM NaF, 5 mM NaVO<sub>3</sub>, and 100  
µg/mL PMSF). Lysates were cleared by centrifugation at 13,000 rpm for 30  
5 minutes. E17-E19 pregnant rats (Long Evans strain) were purchased from Harland  
Sprague-Dawley. P0 pups were harvested and their cortices were dissected and  
15 cultured as previously described<sup>6</sup>. Cortical cultures were grown in 24 well plates on  
either plastic or glass coverslips (400,000 cells/ per well) that had been treated with  
laminin and poly-D-lysine. Cultures were maintained in basal growth media (BGM)  
20 for 3 days prior to virus infection. Swiss 3T3 and COS-7 cells were maintained in  
DMEM supplemented with 10% fetal calf serum. Ac-DEVD-CHO was added to  
media (10µM) approximately 8 hours before infection and then every day (10µM)  
after infection until cells were harvested.

#### 25 Transfection of COS-7 cells, cycloheximide treatment and kinase Assays

15 COS-7 were transiently transfected with various plasmid constructs using  
calcium phosphate transfection procedures. Amounts of CMV plasmid DNA used  
30 are as follows: HIS<sub>6</sub>-hTau40 (5 µg), p35 (5 µg), p25 (2.5 µg), cdk5 (5 µg), DNK5  
(5µg), and HA-GSK3-β (10 µg); for another experiment the following was used: 5x  
as much p35 than p25 plasmid DNA. Cycloheximide t<sub>1/2</sub> experiments were  
35 20 performed. Histone H1 kinase activity was determined as follows; ~ 1 mg of protein  
from brain lysates or transfected cell lysates was immunoprecipitated with p35 (4E3)  
or cdk5 (C8) antibodies, respectively. Histone H1 was added as a substrate in the *in*  
40 *vitro* kinase assay performed as previously described. In the case of p25  
phosphorylation by cdk5, no histone H1 was added. Subcellular fractionation was  
25 done as previously described.

#### 45 Immunohistochemistry

P0 cortical cultures three days in culture were infected with various  
combination of HSV- βgal, HSV-p35, HSV-p25, HSV-GFP-cdk5, and HSV-GFP-  
50 DNK5. 2-3 days after infection cultures were fixed with acetone:methanol (1:1) for  
30 3 min at room temp (RT), washed 3X with PBS and permeabilized with 0.2% triton

5 X-100 in blocking solution ( 1% blocking reagent (Boehringer Mannheim) in PBS)  
for 30 min at RT. Cultures were washed 3X with PBS and incubated with either  
10 PHF-1 (1:100), AT8 (1:100) , p35 (1:400), or  $\beta$ gal (1:400) for 12 hr in blocking  
solution. The cultures were then washed 3X with PBS and rabbit or mouse  
5 secondary biotinylated antibodies were added (1:150) in blocking solution and  
detected by Vector Elite substrate kit (Vector, Burlingame, CA). Diaminobenzidine  
15 (DAB- brown) and Vector SG substrate was used in double labeling experiments.  
Double labeling was performed sequentially.

Calcium phosphate transfection of cortical cultures (E17-E19; two days in  
20 culture) were performed as previously described. A CMV- $\beta$ gal plasmid (10  $\mu$ g) was  
transfected with either CMV-NEO control plasmid(50  $\mu$ g), CMV-p35 and CMV-  
cdk5 (25  $\mu$ g each), CMV-p25 and CMV-cdk5 (25  $\mu$ g each), or CMV-HA-GSK3- $\beta$   
25 (50  $\mu$ g). 2-3 days after transfection the cells were fixed and stained with  $\beta$ gal  
antibodies and Hoechst dye.

15 Transfected Swiss 3T3 cells were fixed with 4% paraformaldehyde and  
permeabilized in 0.3% triton X-100. Immunostaining was done as previously  
described<sup>6</sup> using anti-HA (mAb 12CA5), anti-p25 (pAb neu-cyc, pAb N-20: Santa  
30 Cruz), anti-actin (mAb: Company), to detect p35, p25, p35 G2A, HA-p10, and actin.  
The cells were then treated with biotin-conjugated anti-rabbit (followed by FITC-  
20 conjugated streptavidin) and Texas-Red-conjugated mouse antibodies. Coverslips  
35 were mounted in ProLong antifade (Molecular Probes) and analyzed using a Leica  
or Zeiss confocal microscope. Where stated, normal fluorescent microscopy was  
used.

40 For staining of control and AD brain tissue; paraffin sections (8 microns  
25 thick) were de-waxed in xylenes, hydrated through graded alcohol solutions, and  
blocked with 3% BSA, 10% NGS, and 0.1% Triton X-100. The sections were  
incubated in citrate buffer for 10 minutes at 95°C for antigen retrieval. The sections  
45 were incubated for 1 hour at room temp. or overnight at 4°C with 1 to 4  $\mu$ g/ml of  
primary antibody. Bound rabbit and mouse antibodies were detected using  
30 Vectastain Elite Avidin-Biotin kit (Vector, Burlingame, CA) with diaminobenzidine  
(DAB) or Vector®SG as substrate. For double labeling studies, binding of the first  
50 primary antibody was detected as described, and binding of the second primary

antibody was detected using DAB or Vector®SG as substrate. At the conclusion of the immunostaining reactions, the sections were dehydrated and mounted with Permount® (Fisher Scientific) under coverglass..

A modified silver stain was used. Infected cultures were fixed with 90% ethanol, 5% formaldehyde, and 5% acetic acid prior to silver staining procedures.

#### Microtubule Binding Assay

Tau's binding to taxol stabilized microtubules (MAPs free tubulin - Cytoskeleton) was assayed. Briefly, approximately 1 mg of 200,000xg cell lysates (lysed in 100 mM PIPES, pH 6.8, 0.1% Triton X-100, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM GTP, plus inhibitors) from transfected cells were incubated with 100 µg taxol polymerized microtubules at 35°C for 30 min. The microtubule bound proteins were separated by centrifugation at 70,000 xg for 30 min. Pellets were resuspended in same volume as supernatants and both bound and unbound fractions were analyzed by western blot analysis.

#### Western Blot analysis

P0 cortical cultures, brain lysates, and transfected cells were washed with PBS and lysed with RIPA buffer plus inhibitors ( 2 µg/mL of aprotinin, 2 µg/mL of leupeptin, 1 µg/mL of pepstatin, 5 mM NaF, 5 mM NaVO<sub>3</sub>, and 100 µg/mL PMSF). In some cases, samples were immunoprecipitated with either p35 (polyclonal) or cdk5(C8) antibodies. Sample buffer was added to lysates and immunoprecipitates and samples were run on SDS-PAGE gels (see text and figure legends for gel percentage), electrotransferred to nylon membrane and probed with either Tau-1 (1:2000), PHF-1 (1:1000), AT-8 (1:1000), beta-tubulin (1:2000), anti-6X-HIS (1:2000), ant-HA (12CA5 - 1:25), DC17 (1:20), or p35 (C19- Santa Cruz- 1:2000, p35 GST-p25 purified polyclonal - 1:2000, and N-terminal -1:1000).

#### Cell Death Measurements and DNA Laddering

After immunostaining, neurons or COS-7 cells were labeled with DNA dye Hoechst 33258 (2.5 µg/mL, 5 min), and infected neurons or transfected COS-7 cells were scored for healthy or apoptotic nuclear morphology. Cells were scored

5 positive if they had a pyknotic and/or fragmented nucleus. Representative graphs  
are presented for experiments where 150 or 300 cells were scored. All experiments  
10 were done at least three separate times. TUNEL assays were done according to  
standard procedure (Boehringer Mannheim). For DNA laddering - cells were lysed  
5 and soluble fragmented DNA purified. DNA was run on a 1.5% agarose gel.

15  
Example 4: Additional Data Supporting the role of p25

20 A 25 kDa proteolytic product of p35 is accumulated in Alzheimer's disease brain  
The expression profiles of p35 and cdk5 from human brain tissues were  
surveyed. While p35 levels remained relatively constant in all samples, a 25 kDa  
10 species, recognizable by anti-p35 antibodies, was found to be accumulated 20-40  
25 fold that of p35 in all but one Alzheimer's disease (AD) sample. Cdk5 levels do not  
vary significantly between normal and AD samples. A patient with terminal stage of  
AD was studied and had a large loss of brain tissue (brain weight of 950 grams),  
30 which may account for the lack of accumulation of the 25 kDa species. To verify  
15 the identity of the 25 kDa species, antibodies recognizing various regions of p35  
were utilized. p35 is the most prominent protein recognized by these antibodies in  
rat brain lysate as detected by western blot analysis. The accumulation of this 25  
35 kDa species in AD samples corresponded to the elevated cdk5 kinase activity in the  
AD samples as indicated by the cdk5 associated histone H1 kinase activity. As p35  
20 is phosphorylated in a cdk5 immunoprecipitation/kinase assay, the 25 kDa species  
was found to be similarly phosphorylated. This 25 kDa species was contained in  
40 immunocomplexes that were immunoprecipitated with either cdk5 antibody or  
antibodies recognizing the C-terminal portion of p35, indicating that the 25 kDa  
species is either a cleavage product of p35 or derived from a related protein. N-  
45 25 terminal specific p35 antibody did not recognize the 25 kDa species. The size of the  
25 kDa species is reminiscent of p25 previously co-purified with cdk5 from bovine  
brain lysates. Indeed, the 25 kDa species co-migrated with p25 from COS-7 cell  
50 lysates transfected with CMV-p25 (p35 N-terminal deletion mutant generated  
according to the published sequences) on SDS-PAGE. To determine if the 25 kDa



5 species observed in AD brains lysates is actually p25, the V8 digest pattern of the  
AD 25 kDa species was compared to that of p25 derived from COS-7 cell lysate  
10 transfected with CMV-p25 which were observed identical. Based on the  
observations that the 25 kDa species is recognized by the p35 C-terminal specific  
5 antibodies but not by the N-terminal specific antibodies, that it associates with cdk5  
and with cdk5 kinase activity, that it co-migrates with p25 on SDS-PAGE and that it  
15 displays an identical V8 digestion pattern as that of recombinant p25, the 25 kDa  
species accumulated in AD brains is indeed p25. As p35 is encoded by one exon  
without interruption by introns, alternative splicing cannot account for the  
20 production of p25. Rather, p25 is most likely to be generated by proteolytic  
cleavage at a specific site.

25 p25 is present in neurons containing neurofibrillary tangles in Alzheimer's disease  
brain:

Since the p25 protein levels were vastly elevated in AD brain lysates, it was  
15 of interest to determine the distribution of p25 immunoreactivity in histological  
sections from AD and normal brain. The p25 polyclonal antibody was found to  
30 label certain neurons in AD sections taken from the hippocampal formation. This  
antibody otherwise decorated the periphery of the perikaryon of hippocampal  
neurons in the AD and control sections. Adjacent sections were stained with AT8,  
35 an anti-phospho-tau antibody, which labeled neurons containing neurofibrillary  
tangles (NFT) in AD but not control sections. Double immunostaining with anti-  
p25 and AT8 revealed that many NFT containing neurons were also positive for  
40 p25. However, there were more neurons with elevated p25 immunoreactivity than  
neurons displaying NFTs. Immunostaining was also carried out with anti-p10  
25 antibody which only detects full length p35. Anti-p10 also decorated the neuronal  
periphery in the control section; its immunoreactivity did not accumulate in neurons  
45 containing NFT. Together, western blot analysis and immunohistochemistry of AD  
tissues indicates that p25, but not p35, is accumulated in AD and is present in  
neurons affected by neurodegeneration.

50 30 p25 is distinct from p35 in subcellular distribution and stability

5 p25 contains all the elements required for cdk5 activation and was shown to  
activate cdk5 *in vitro*. As p25, but not p35, is accumulated in AD, it was determined  
10 whether these two proteins display different biochemical properties. Both cdk5 and  
p35 are enriched in the processes and growth cones of neurons and that p35  
5 segregates with the plasma membrane. It was found that N-terminal myristoylation  
signal motif is highly conserved in mammalian, *Xenopus*, and *C. elegans* p35  
15 homologues. To determine if this myristoylation signal is required for the normal  
subcellular localization of p35, the conserved glycine at position 2 was mutated to  
alanine and expressed in COS-7 cells. While p35 was localized at the cell periphery  
20 and induced lamellipodia and filopodia structures, the G2A mutant was absent at the  
cell periphery demonstrating that the myristoylation signal is essential for the proper  
distribution of p35. Since p25 lacks the conserved myristoylation sequence, its  
subcellular localization was compared with that of p35 in transfected fibroblasts.  
25 p25 was enriched in nuclear and perinuclear regions of the cell, while p35 and p10  
(an N-terminal fragment of p35 lacking the p25 region) were localized and enriched,  
in the case of p10, at the cell peripheries. Distribution of p25 was further  
30 investigated by subcellular fractionation. While p35 was more abundant in the  
membrane fraction, p25 was enriched in the cytosolic fraction. p35 is normally  
targeted to the membrane *in vivo*. In contrast, p25 which is not targeted to the  
20 plasma membrane, likely sequesters cdk5 away from compartments of the cell where  
p35/ckd5 activity is normally required. In addition, in primary cortical neurons, p25  
35 is primarily concentrated in the cell soma and largely absent in neurites while p35  
has been shown to be present in the peripheries and nerve terminals.

40 In addition to the difference in subcellular localization of p35 and p25, there  
is a vast difference in turnover rate between the two. Our previous studies indicated  
25 that cdk5 activity is tightly regulated by p35 protein levels. p35 was determined to  
have a half-life of ~20 to 30 minutes in primary cortical neurons and this rapid  
45 turnover-rate was in part due to phosphorylation-stimulated, ubiquitin-mediated  
degradation. When expressed in COS-7 cells together with cdk5, p25 had an  
30 approximately 5-10 fold longer half-life than p35 or p35 G2A mutant. Cdk5  
associated histone H1 kinase activity paralleled the levels of p35 or p25. Together,  
50 these observations indicate that the accumulated p25 in AD brains may cause

5 prolonged activation of cdk5 and mis-localization of cdk5 kinase activity in affected neurons.

10 Efficient tau phosphorylation by the p25/cdk5 kinase *in vivo*

To further explore a functional difference between the p35/cdk5 and  
5 p25/cdk5 kinases *in vivo*, the activity of these two kinases were compared in  
15 phosphorylation of the microtubule associated protein tau. Tau was shown to be a  
substrate of cdk5. A 6X-histidine-tagged human tau 40 (htau40) was co-transfected  
with p35/cdk5, p25/cdk5 or p25/DNK5 (a catalytically inactive mutant of cdk5) in  
20 COS-7 cells. Tau phosphorylation was evaluated by immunoblotting with AT8 or  
10 PHF-1. AT8 recognizes phosphorylated tau epitopes at Ser202 and Ser205 and  
PHF-1 recognizes phosphorylated epitopes at and around Thr396, sites previously  
shown to be phosphorylated by cdk5 *in vitro*. Intense AT8 immunoreactivity was  
25 seen in cells expressing p25/cdk5 but not p35/cdk5 or p25/DNK5. Interestingly,  
PHF-1 antibody detected a slower migrating species in the p25/cdk5 expressing cells  
15 which was readily abolished upon protein phosphatase treatment, indicating that this  
slower migrating band was indeed a phosphorylated species of tau. This species was  
30 not present in cells expressing p35/cdk5 or p25/DNK5. As a control, GSK3- $\beta$ , a  
well established kinase for tau, caused a large increase in tau PHF-1  
immunoreactivity.

35 20 Due to the difference in turnover rate, the steady state levels of p35 were  
always much lower than p25, which may have contributed to the observed difference  
in tau phosphorylation. In order to compare the ability of these kinases to  
40 phosphorylate tau when similar levels of the two kinases are expressed *in vivo*, five  
times more p35 than p25 plasmid DNA was used for transfection. However, even  
25 after p35 and p25 levels were expressed at comparable levels, the slower migrating  
PHF-1 immunoreactive species of tau was still absent in p35/cdk5 transfected cells,  
45 despite the fact that an increase in PHF-1 immunoreactivity was observed, indicating  
that other differences such as the altered subcellular distribution of the p25/cdk5  
kinase may allow for more efficient targeting of tau *in vivo*. The levels of tau  
30 expressed were comparable as indicated by immunoblotting with 6X-HIS  
50 antibodies. Interestingly, anti-6X-HIS antibodies also revealed a shift in tau

5 mobility in p25/cdk5 co-transfected cells. Transfection of p25 alone did not result in  
a noticeable increase in PHF-1 or AT8 immunoreactivity and is likely to reflect the  
10 low endogenous level of cdk5 in COS-7 cells. When transfected COS-7 cell lysates  
were incubated with polymerized microtubules, tau's ability to bind microtubules  
5 was impaired in cells co-expressing the active p25/cdk5 kinase complex, indicating  
that indeed the hyperphosphorylation of tau by p25/cdk5 may affect its function *in*  
15 *vivo*.

The p25/cdk5 kinase causes morphological degeneration and cytoskeletal disruption  
of neurons

20 The effects of p25/cdk5 on tau hyperphosphorylation were also investigated  
in primary cortical neurons using a Herpes Simplex recombinant viral expression  
system. After  $\beta$ gal virus infection, weak PHF-1 immunoreactivity was present in  
25 the cell soma as well as axon fibers. In contrast, infection with p25/cdk5 produced  
robust PHF-1 immunoreactivity that was concentrated in the cell soma. This effect  
15 was reversed when p25/DNK5 was expressed indicating that the catalytic activity of  
cdk5 is necessary for the observed increase in PHF-1 signal. p35/cdk5 infected  
30 neurons also displayed increased PHF-1 immunoreactivity but to a lesser extent.  
Similar results were obtained using AT8 antibody. Many p25/cdk5 infected  
neurons, indicated by PHF-1 positive staining, exhibited neurite retraction, whereas  
35 p35/cdk5 infected cultures seldom exhibited signs of neurite degeneration.  
Neurofilaments are also well established substrates of cdk5. Using SMI34, a  
phospho-specific neurofilament H antibody, applicants observed intense phospho-  
neurofilament immunoreactivity in p25/cdk5 infected neurons, but less intense  
40 staining in uninfected neurons, indicating that the p25/cdk5 kinase causes  
25 hyperphosphorylation of neurofilaments.

Tau hyperphosphorylation has been shown to cause microtubule  
destabilization. p25/cdk5 phosphorylated tau bound to microtubules less well.  
45 Thus, a silver-based stain method was utilized to assess the cytoskeletal integrity of  
p25/cdk5 expressing neurons. Silver-positive neurons were frequently seen in the  
p25/cdk5 expressing cultures. Silver labeling was specific to p25/cdk5 infected  
30 neurons and was never observed in  $\beta$ gal, p35/cdk5, or p25/DNK5 infected cultures.  
50

5 Together, these data indicate that p25/cdk5 is more potent than p35/cdk5 in  
phosphorylating protein substrates such as tau and neurofilaments *in vivo*, which  
10 may be, in part, attributable to its accumulation and difference in localization.  
Furthermore, they indicate that the presence and accumulation of the p25/cdk5  
5 kinase is associated with the incidence of morphological degeneration and  
cytoskeletal disruption in neurons, events seen in AD and other neurodegenerative  
15 diseases.

The p25/cdk5 kinase induces profound apoptotic cell death in neurons

20 In order to further characterize cytoskeletal abnormalities,  $\beta$ -tubulin in the  
infected cultures was stained.  $\beta$ -tubulin staining revealed a drastic difference in the  
10 microtubule network in p25/cdk5 versus p25/DNK5 or  $\beta$ gal control infected cells.  
Tubulin was normally distributed throughout the cell soma as well as axonal and  
25 dendritic compartments, as seen in  $\beta$ gal and p25/DNK5 infected cultures. In  
contrast, tubulin was concentrated in the perikarya of p25/cdk5 infected cells. In  
15 fact, most of the p25/cdk5 infected cells were devoid of neurites. The presence of  
apoptotic cell bodies was frequently observed in p25/cdk5 infected cultures, but not  
30 in  $\beta$ gal or p25/DNK5 infected cultures.

Additionally applicants noticed in COS-7 cell transfection experiments that  
many cells died upon co-expression of p25 and cdk5. The extent of cell death in  
35 COS-7 cells transfected with either p25/cdk5 or p35/cdk5 was compared by  
purifying soluble fragmented DNA from these cells. DNA laddering was evident in  
20 p25/cdk5 transfected cells which was present at a much reduced level in p35/cdk5 or  
the empty vector control transfected cells. Nuclear morphology of infected cells was  
40 further examined by Hoechst stain.  $\beta$ gal infected neurons had normal nuclear  
25 morphology, whereas, the majority of p25/cdk5 infected neurons had fragmented  
and condensed nuclei. Moreover, most of the p25/DNK5 infected neurons had  
45 normal nuclear morphology. Neurons which displayed fragmented nuclei were also  
positive for TUNEL staining. In general, more than 85% of p25/cdk5 infected  
neurons had fragmented or condensed nuclear morphology and the effect was  
50 largely reversed by the expression of DNK5. Very few neurons had nuclear

5 fragmentation after  $\beta$ gal viral infection. Approximately 30% of p35/cdk5 infected  
neurons displayed disrupted nuclei. These results were corroborated by calcium  
10 phosphate transfection of primary cortical neurons. A  $\beta$ gal DNA construct was co-  
transfected with various plasmid DNA at a ratio of 1:5 to ensure that most positively  
5 scored  $\beta$ gal cells expressed the genes of interest. Approximately 65-70% of  
p25/cdk5 transfected neurons had fragmented nuclei, whereas less than 5% in  $\beta$ gal  
15 alone and approximately 20% in p35/cdk5 transfected neurons had abnormal nuclear  
morphology. Additionally, the p35 G2A mutant did not cause significant cell death.  
GSK3- $\beta$  expression caused no detectable abnormality in nuclear morphology in  
20 primary cortical neurons. The p25/cdk5 induced nuclear  
condensation/fragmentation could be partially inhibited by Ac-DEVD-CHO, an  
inhibitor of caspase-3.

25 The apoptotic cell bodies revealed by tubulin staining, DNA laddering and  
fragmented and condensed nuclear morphology provide compelling evidence for a  
15 pro-apoptotic effect of the p25/cdk5 kinase. The inability of the catalytically  
inactive cdk5 mutant to induce apoptosis indicates that substrate phosphorylation is  
necessary for cell death to occur. The much reduced apoptotic cell death with  
30 p35/cdk5 over-expression supports the notion that deregulation of cdk5 is  
detrimental to cells. In addition to the increase in kinase level, p25 mediated mis-  
20 localization of cdk5 activity also contributes to the observed degeneration in  
neurons.

#### Discussion

40 In summary, a proteolytic fragment of p35, p25, accumulates in AD brains  
and present in neurons displaying neurofibrillary tangles. While p35 is required for  
25 normal brain development, the presence of p25 causes deregulation of cdk5 kinase  
activity due in part to the fact that p25 is a stable protein and that it is  
45 inappropriately localized. Other evidence indicates that the N-terminal portion of  
p35 may be necessary for binding to regulatory proteins. Therefore, it is  
conceivable that in neurons containing high levels of p25, cdk5 is sequestered from  
30 normal regulation and concentrated at abnormal site(s), and phosphorylates

5 substrates not normally phosphorylated (or hyperphosphorylated) by this kinase.  
For instance, the p25/cdk5 kinase displays an increased and altered tau  
10 phosphorylation in comparison to the p35/cdk5 kinase *in vivo*. Furthermore,  
neurofilament H is also heavily phosphorylated in p25/cdk5 expressing neurons.  
5 Numerous reports indicate that hyperphosphorylation of MAPs, such as tau, alters  
their interactions with microtubules and causes microtubule instability. Indeed, tau  
15 phosphorylated by the p25/cdk5 kinase displays reduced binding to microtubules. In  
addition, p25/cdk5 expressing neurons displayed neurite retraction and showed signs  
of microtubule collapse. Many of them could be labeled with a silver-based staining  
20 method which is commonly used as an indicator of cytoskeletal disruption. Thus,  
deregulation of cdk5 by the accumulation of p25 impairs the integrity of the  
cytoskeleton which ultimately results in morphological degeneration and, perhaps,  
apoptosis of neurons. Morphological degeneration and neuronal death are  
25 fundamental aspects of many neurodegenerative diseases. Moreover, our findings  
are nicely corroborated by the findings by others, where the p35/cdk5 kinase was  
shown to be associated with neuronal death. Other possible deleterious effects of  
30 p25 are that it may prevent the p35/cdk5 kinase from phosphorylating its normal  
substrates such as Pak1, synapsin, syntaxin, Munc18, and DARP32 by sequestering  
cdk5 from cell periphery and nerve terminals, which also contributes to neuronal  
20 dysfunction.

35 Taken together, conversion of p35 to p25 results in deregulation of the cdk5  
kinase. The deregulated cdk5 kinase can cause irreversible damage to the  
cytoskeleton and neuronal death. Based on the accumulation of p25 in AD brains  
and the cytoskeletal disruption and apoptosis induced by the p25/cdk5 kinase in  
40 neurons, p25 production and accumulation in brain may contribute to the  
pathogenesis of AD. p25 contributes to early stages of AD as it was not  
accumulated in a terminal stage AD patient with significant cell loss. Furthermore,  
45 neurons with elevated p25 outnumbered neurons containing NFT in AD sections.  
Thus, p25 accumulation may precede the formation of NFT. Conversion of p35 to  
30 p25 is likely to be the consequence of proteolytic cleavage and our preliminary  
results indicate that cleavage of p35 to p25 can be activated upon oxidative stress. It  
50 will be of great interest to identify the putative protease that cleaves p35, as it may

5 serve as a therapeutic target for prevention and treatment of neurodegenerative diseases.

10 Example 5: Additional Data Supporting Discovery and Function of the Protase That Cleaves p35

15 5 Applicants have shown an accumulation of a proteolytic cleavage product of p35, p25, in neurodegenerative brains containing neurofibrillary tangles. p25, but not p35, caused efficient tau hyperphosphorylation and apoptotic cell death. These results indicate that cleavage of p35 to p25 plays a role in the pathogenesis of neurodegeneration and this it is of central importance to identify the protease that 20 cleaves p35 to produce p25. The calcium dependent protease calpain is responsible for p35 cleavage. In fresh brain lysates, addition of millimolar range of calcium ion allows p35 cleavage to occur. Inhibitors to calpain completely inhibit the appearance of p25. Furthermore, purified calpain can digest p35 to produce p25 when incubated together. In primary cortical cultures, activation of calpain by 25 ionomycin treatment allows complete conversion of p35 to p25. Finally, global ischemic treatment of mouse brains induced conversion of p35 to p25, and calpain is known to be activated during ischemia. Based on these results, calpain is the protease that cleaves p35 to produce p25.

30 Calcium ion stimulates the conversion of p35 to p25

35 20 To elucidate the molecular events leading to the conversion of p35 to p25, an *in vitro* p35 conversion assay was set up in which mouse brain lysate was incubated with different kinds of inorganic ions. Of the ions tested, calcium ion strongly stimulates the conversion of p35 to p25. Magnesium ion can also stimulate the conversion of p35 to p25, but it is not as effective as calcium ion. These data 40 indicate the existence of a factor in the mouse brain lysate that can cleave p35 to produce p25, and the activity of this factor is dependant on the levels of calcium and magnesium ions.

45 To determine the amount of calcium ion required to stimulate p35 conversion, different concentrations of calcium ion were added to the mouse brain 50 lysate. p25 was only produced when millimolar concentration of calcium ion was



5 present, but not when the concentration of calcium ion was at micromolar level.

10 Calcium stimulated p35 conversion can be inhibited by calpain inhibitors

Calpain is a protease that is activated by a cation, namely, a calcium ion. To determine if calpain is involved in the conversion of p35 to p25, the calpain

5 inhibitors calpeptin and ALLM were added to calcium-treated mouse brain lysate.

15 Both calpeptin and ALLM completely inhibited the conversion of p35 to p25, indicating that calpain plays an important role in the conversion process.

20 Both u-calpain and m-calpain can cleave p35 to produce p25

To determine whether calpain directly cleaves p35, or whether calpain  
10 activates some other factors to cleave p35, either purified u-calpain or m-calpain was incubated with p35 immunoprecipitated from mouse brain lysate using a p35  
25 antibody. Both purified u-calpain and m-calpain can cleave p35 to produce p25, indicating that calpain is likely to be directly cleaving p35.

30 The conversion of p35 to p25 can be stimulated in cortical neuronal culture by

15 ionomycin treatment

To induce the conversion of p35 to p25 in neuronal culture, primary rat  
cortical neurons were treated with ionomycin. Ionomycin is a calcium ionophore  
35 that can induce calcium influx in many cell types. The elevated intracellular ionophore that can induce calcium influx in many cell types. The elevated  
20 intracellular calcium level due to ionomycin treatment is expected to activate calpain, which is expected to then cleave p35 to form p25. Indeed, when the  
40 primary neuronal culture was treated with 10uM ionomycin for four hours, all the endogenous p35 was converted to p25.

45 The conversion of p35 to p25 occurs during ischemic brain damage

25 During ischemic brain damage, neurons experience a sustained elevation of intracellular calcium level due to the perturbation of intracellular calcium homeostasis. To determine whether p35 cleavage occurs during ischemia, intact  
50 adult mouse brains were challenged with a global ischemia condition and assayed

5

the level of p25 in the brain lysate. Very little p25 was seen in the control brains, while a substantial amount of p35 was converted to p25 in the ischemia challenged brains, indicating that p35 is cleaved to form p25 during ischemic brain damage.

10

The relevant teachings of all the references, patents and/or patent applications cited herein are incorporated herein by reference in their entirety.

15

#### EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

20

10

25

30

35

40

45

50

55

**Claims**

5

10

15

20

25

30

35

40

45

50

55

## CLAIMS

What is claimed is:

1. A method of preventing or treating a neurodegenerative disease in an individual, comprising administering to the individual an amount of one or more compounds that reduce conversion of p35 to p25 in neurological tissue.
2. The method of Claim 1, wherein the compound that reduces conversion of p35 to p25 is a compound that inhibits calpain or calcium.
3. The method of Claim 2, wherein the method further includes administering p35 to the individual.
4. The method of Claim 3, wherein a modified form of p35 is administered.
5. The method of claim 1, wherein the neurodegenerative disease is associated with neurofibrillary tangles and accumulation of p25 in the brain.
6. The method of Claim 5, wherein the neurodegenerative disease is selected from the group consisting of: dementias, neurodegenerative diseases associated with stroke, neurodegenerative diseases associated with myocardial infarction, neurodegenerative diseases associated with ischemia, and neurodegenerative diseases associated with other oxidative stresses.
7. The method of Claim 6, wherein the neurodegenerative disease is Alzheimer's disease, Corticobasal degeneration, Dementia pugilistica, Down's syndrome, Frontotemporal dementias and Parkinsonism linked to chromosome 17, Myotonic dystrophy, Niemann-Pick disease, Parkinson-dementia complex of Guam, Pick's disease, postencephalic Parkinsonism, prion disease with tangles, progressive supranuclear palsy, lower lateral sclerosis, Huntington's Disease, subacute sclerosing panencephalitis and

multiple sclerosis.

8. A method of preventing or treating a neurodegenerative disease in an individual, wherein the neurodegenerative disease is associated with neurofibrillary tangles and accumulation of p25 in neurological tissue, comprising administering to the individual an amount of one or more compounds that inhibit deregulation of a cdk5 kinase.
9. The method of Claim 8, wherein the compound inhibits association of p25 and cdk5.
10. The method of Claim 9, wherein the compound inhibits calpain or calcium.
11. The method of Claim 10, wherein the neurodegenerative disease is selected from the group consisting of: dementias, neurodegenerative diseases associated with stroke, neurodegenerative diseases associated with myocardial infarction, neurodegenerative diseases associated with ischemia, and neurodegenerative diseases associated with other oxidative stresses.
12. The method of Claim 11, wherein the neurodegenerative disease is Alzheimer's disease, Corticobasal degeneration, Dementia pugilistica, Down's syndrome, Frontotemporal dementias and Parkinsonism linked to chromosome 17, Myotonic dystrophy, Niemann-Pick disease, Parkinson-dementia complex of Guam, Pick's disease, postencephalic Parkinsonism, prion disease with tangles, progressive supranuclear palsy, lower lateral sclerosis, Huntington's Disease, subacute sclerosing panencephalitis and multiple sclerosis.
13. A method of preventing or treating a neurodegenerative disease in an individual, wherein the neurodegenerative disease is associated with neurofibrillary tangles and accumulation of p25 in neurological tissue, comprising administering to the individual an amount of one or more

compounds that reduce phosphorylation of tau by a p25/ckd5 kinase.

14. The method of Claim 13, wherein the compound inhibits association of p25 and cdk5.

15. The method of Claim 14, wherein the compound inhibits calpain or calcium.

16. A method of treating a neurodegenerative disease in an individual, wherein the neurodegenerative disease is associated with neurofibrillary tangles and accumulation of p25 in the brain, comprising administering to the individual an amount of one or more compounds that reduce accumulation of p25 in the brain.

17. The method of Claim 16, wherein the compound inhibits calpain or calcium.

18. A method of preventing or treating a neurodegenerative disease in an individual comprising administering to the individual an effective amount of one or more calpain inhibitors or antagonists, wherein the inhibitors or antagonists reduce conversion of p35 to p25.

19. The method of Claim 18, wherein the calpain inhibitor or antagonist is selected from the group consisting of: calpeptin, N-acetyl-leucyl-leucyl-methional (ALLM), N-acetyl-leucyl-leucyl-norleucinal (ALLN), calpastatin, AK275, MDL28170, E64, leupeptin and calpain inhibitor I.

20. The method of Claim 19, wherein the neurodegenerative disease is selected from the group consisting of: Alzheimer's disease, Corticobasal degeneration, Dementia pugilistica, Down's syndrome, Frontotemporal dementias and Parkinsonism linked to chromosome 17, Myotonic dystrophy, Niemann-Pick disease, Parkinson-dementia complex of Guam, Pick's disease, postencephalic Parkinsonism, prion disease with tangles, progressive supranuclear palsy, lower lateral sclerosis, Huntington's Disease, subacute

sclerosing panencephalitis and multiple sclerosis.

21. The method of preventing or treating a neurodegenerative disease in an individual comprising administering to the individual one or more cation antagonists or inhibitors, wherein the antagonists or inhibitors reduce conversion of p35 to p25.

22. The method of Claim 21, wherein the cation antagonist or inhibitor is a calcium antagonist or inhibitor, or magnesium antagonist or inhibitor.

23. The method of Claim 22, wherein the calcium inhibitor is selected from the group consisting of: MKA01, omega-conotoxin and Sb201823-A.

24. A method of inhibiting or reducing conversion of p35 to p25 in neuronal tissue comprising contacting one or more calpain inhibitors or antagonists, and/or one or more cation inhibitors or antagonists with the neuronal tissue.

25. The method of Claim 24, wherein the neuronal tissue is brain tissue or spinal cord tissue.

26. The method of Claim 25, wherein the calpain inhibitor is selected from the group consisting of: calpeptin, N-acetyl-leucyl-leucyl-methionine (ALLM), N-acetyl-leucyl-leucyl-norleucine (ALLN), calpastatin, AK275, MDL28170, E64, leupeptin and calpain inhibitor I.

27. The method of Claim 26, wherein cation antagonist or inhibitor is a calcium antagonist or inhibitor, or magnesium antagonist or inhibitor.

28. A method of preventing or reducing neurofibrillary tangles comprising contacting one or more calpain inhibitors or antagonists, and/or one or more cation inhibitors or antagonists with neuronal tissue, wherein conversion of p35 to p25 is reduced.

5

10

15

20

25

30

35

40

45

50

55

29. The method of Claim 28, wherein the neuronal tissue is brain tissue or spinal cord tissue.

30. The method of Claim 29, wherein cation antagonist or inhibitor is a calcium antagonist or inhibitor, or magnesium antagonist or inhibitor.

5 31. A method preventing or treating an individual having a neurodegenerative disease comprising administering an amount of one or more calpain inhibitors or antagonists and at least one other composition used for preventing or treating neurodegenerative disease.

10 32. The method of Claim 31, where the other composition used for treating neurodegenerative disease is selected from the group consisting of: COMT inhibitors, non-ergot DE dopamine agonists, monoamine oxidase inhibitors and ropinirole hydrochloride.

15 33. A method of determining the presence or absence of a neurodegenerative disease in an individual, comprising determining the presence or absence of p25 in a sample obtained from the individual, wherein the presence of p25 in the sample as compared to a control indicates the presence of the neurodegenerative disease, and the absence of p25 as compared to a control indicates the absence of a neurodegenerative disease.

20 34. The method of Claim 33, wherein the neurodegenerative disease is selected from the group consisting of: dementias, neurodegenerative diseases associated with stroke, neurodegenerative diseases associated with myocardial infarction, neurodegenerative diseases associated with ischemia, and neurodegenerative diseases associated with other oxidative stresses.

25 35. The method of Claim 34, wherein the neurodegenerative disease is Alzheimer's disease, Corticobasal degeneration, Dementia pugilistica,



Down's syndrome, Frontotemporal dementias and Parkinsonism linked to chromosome 17, Myotonic dystrophy, Niemann-Pick disease, Parkinson-dementia complex of Guam, Pick's disease, postencephalic Parkinsonism, prion disease with tangles, progressive supranuclear palsy, lower lateral sclerosis, Huntington's Disease, subacute sclerosing panencephalitis and multiple sclerosis.

36. A method of determining the presence or absence of a neurodegenerative disease in an individual, comprising:
- obtaining a sample from an individual to be tested;
  - assessing the levels of p25 and p35 in the sample; and
  - comparing the levels assessed to a standard or control,
- wherein an increased level of p25 and a decreased level of p35 indicates relative to a standard indicates the presence of the neurodegenerative disease, and a decreased level of p25 and an increased level of p35 indicates relative to a standard indicates the absence of the neurodegenerative disease.
37. The method of Claim 36, further including forming a ratio of p25 and p35.
38. A method of diagnosing or aiding in the diagnosis a neurodegenerative disease in an individual, comprising:
- determining the presence, absence or level of p25 in a sample obtained from the individual; and
  - comparing the level of p25 determined with a control or standard; wherein a presence or increased level of p25 in the sample indicates the presence of the neurodegenerative disease, and the absence or decreased level of p25 indicates the absence of a neurodegenerative disease.
39. A method of determining the efficacy of treatment for an individual having a neurodegenerative disease, comprising:
- determining the level of p25 in a sample obtained from the individual; and

5  
10 b) comparing the level of p25 determined with a control or standard;  
wherein an increased level of p25 in the sample indicates ineffective  
treatment, and a decreased level of p25 indicates effective treatment.

15 40. The method of claim 40, wherein the level of p35 is determined, and an  
5 increase in the level of p35 as compared to a standard indicates effective  
treatment, and a decrease of the level of p35 as compared to a standard  
indicates ineffective treatment.

20 41. A method of reducing the extent to which a neurodegenerative disease that is  
associated with neurofibrillary tangles and accumulation of p25 in the brain  
10 occurs in an individual, comprising administering to the individual a  
compound that reduces conversion of p35 to p25 in the brain.

25 42. A compound for the prevention or treatment of a neurodegenerative disease,  
comprising a compound selected from the group consisting of:  
30 a) a compound that inhibits the association of p25 with cdk5;  
15 b) a compound that inhibits deregulation of cdk5 by p25;  
c) a compound that reduces the conversion of p35 to p25;  
d) a compound that reduces the phosphorylation of tau by p25/cdk5  
35 kinase;  
e) a compound that inhibits calpain;  
20 f) a compound that inhibits a cation; and  
g) a compound that is an agonist of p35.  
40

45 43. The compound of Claim 42, wherein the compound is an antibody or  
antibody fragment that is specific to p25.

50 44. The compound of Claim 42, wherein the compound is an antibody or  
25 antibody fragment that is specific to cdk5.

55 45. The compound of Claim 44, wherein the compound is a polypeptide.

5

46. A nucleic acid construct encoding a compound of Claim 42.

10

15

20

25

30

35

40

45

50

55

FIG. 1A

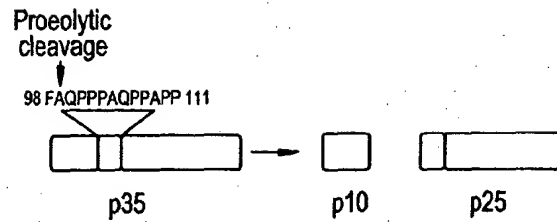


FIG. 1B

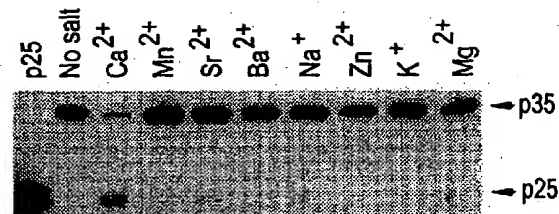


FIG. 1C

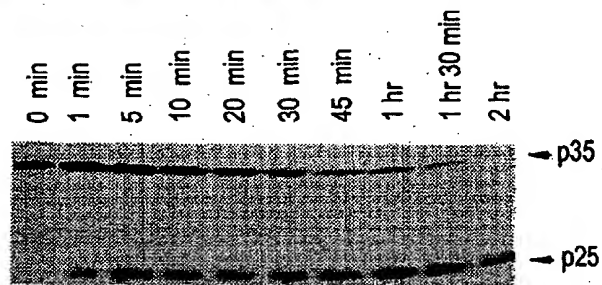


FIG. 1D

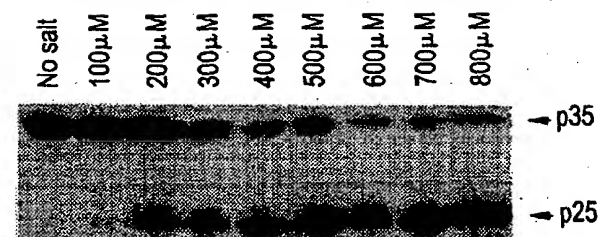


FIG. 2A

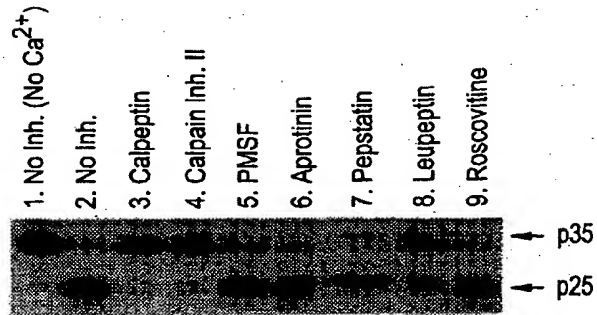


FIG. 2B

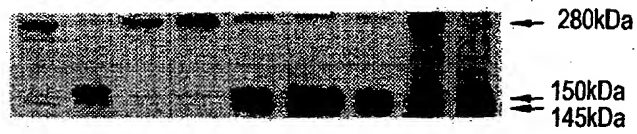


FIG. 2C

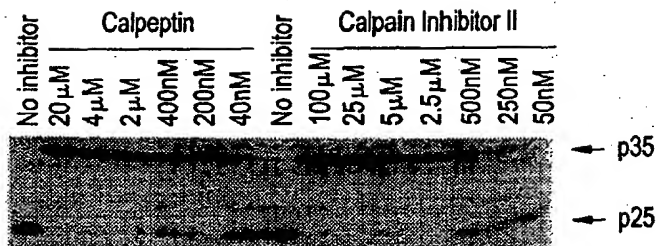


FIG. 2D

Fraction #  
3 4 5 6 7 8 9 10 11 12 13 14 15 16

← p35

← p25

FIG. 2E



← 280kDa

← 150kDa

FIG. 2F

$\mu$ - calpain					m - calpain				
Buffer alone	1 min	5 min	10 min	20 min	Buffer alone	1 min	5 min	10 min	20 min



← p35

← p25

4/6

FIG. 3E

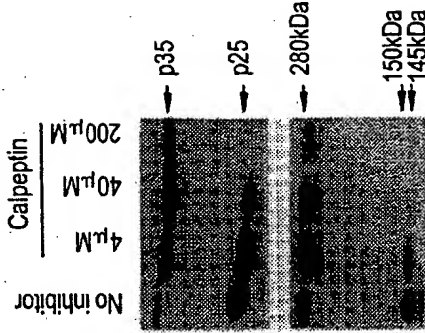


FIG. 3D

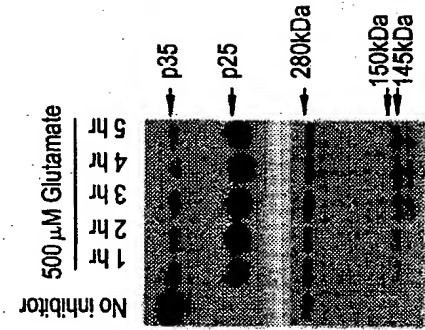


FIG. 3B

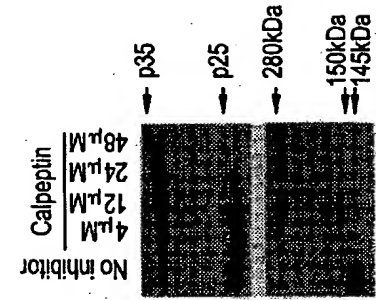


FIG. 3A

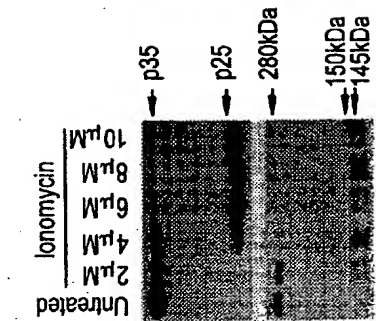


FIG. 3F

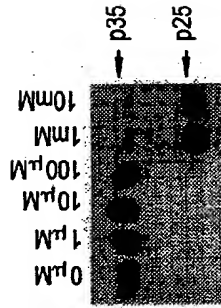
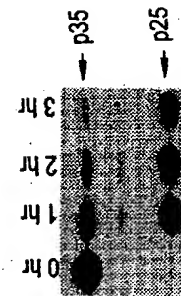


FIG. 3C



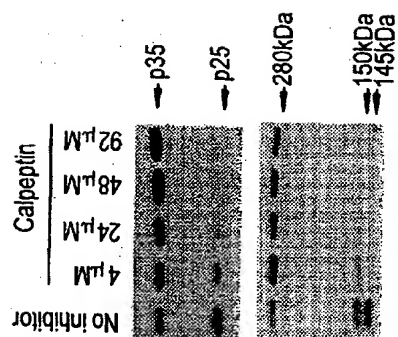


FIG. 4B

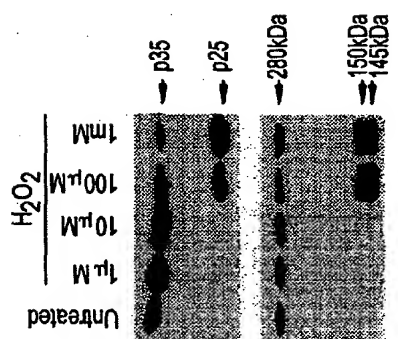


FIG. 4A

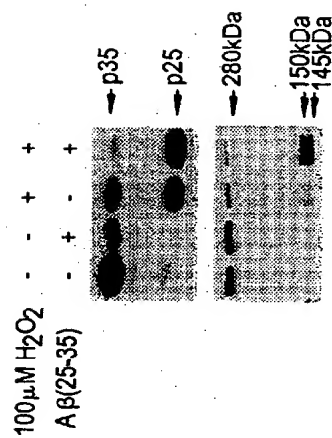


FIG. 4D

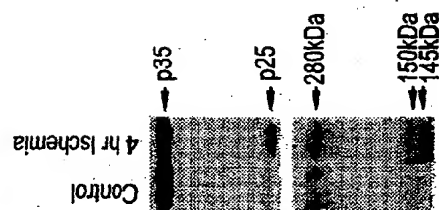


FIG. 4C



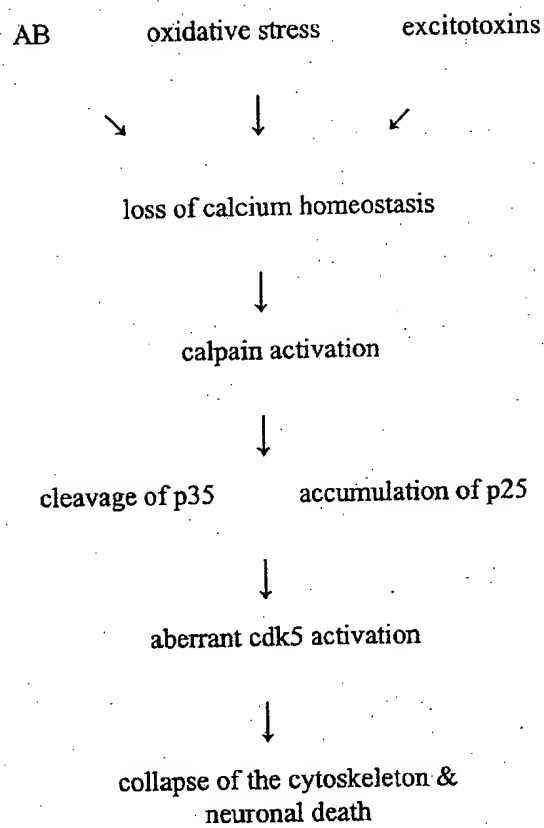


FIG. 4E

SUBSTITUTE SHEET (RULE26)



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: <b>A61K 38/00</b>	<b>A3</b>	(11) International Publication Number: <b>WO 00/21550</b> (43) International Publication Date: 20 April 2000 (20.04.2000)
(21) International Application Number: PCT/US99/24221 (22) International Filing Date: 13 October 1999 (13.10.1999) (30) Priority Data: 60/103,975      13 October 1998 (13.10.1998) US 60/136,631      27 May 1999 (27.05.1999) US (60) Parent Application or Grant PRESIDENT AND FELLOWS OF HARVARD COLLEGE [/]; (). TSAI, Li-Huei [/]; (). PATRICK, Gentry, N. [/]; (). LEE, Ming, Sum [/]; (). BROOK, David, E. ; ().	Published	
(54) Title: METHODS AND COMPOSITIONS FOR TREATING NEURODEGENERATIVE DISEASES (54) Titre: METHODES ET COMPOSITIONS DE TRAITEMENT DES MALADIES NEURODEGENERATIVES  (57) Abstract <p>The present invention relates to methods of preventing or treating neurodegenerative diseases by administering an antagonist or inhibitor of p25. In particular, the invention relates to methods of preventing or treating a neurodegenerative disease by administering a calpain antagonist or inhibitor, or a cation antagonist or inhibitor, which reduces the truncation or conversion of p35 to p25. It also relates to p.35 and antibodies directed to p.25 and cdk 5. It relates to methods of inhibiting p25/cdk association, cdk 5 deregulation and phosphorylation of tan. Methods of assessing neurodegeneratives diseases using p 25 are provided.</p> (57) Abrégé <p>La présente invention se rapporte à des méthodes de prévention ou de traitement des maladies neurodégénératives consistant à administrer un antagoniste ou un inhibiteur de p25. L'invention se rapporte notamment à des méthodes de prévention ou de traitement d'une maladie neurodégénérative, qui consistent à administrer un inhibiteur ou antagoniste de calpaïne, ou un inhibiteur ou antagoniste de cations, qui réduit la troncature ou la conversion de p35 en p25.</p>		

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>A61K 38/55, 38/05, 38/17, 31/00,</b> <i>(Continued on the following page)</i>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/21550</b> <b>(43) International Publication Date:</b> 20 April 2000 (20.04.00)
<b>(21) International Application Number:</b> PCT/US99/24221 <b>(22) International Filing Date:</b> 13 October 1999 (13.10.99)  <b>(30) Priority Data:</b> 60/103,975 13 October 1998 (13.10.98) US 60/136,631 27 May 1999 (27.05.99) US  <b>(71) Applicant:</b> PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; Holyoke Center, Suite 727, 1350 Massachusetts Avenue, Cambridge, MA 02138 (US).  <b>(72) Inventors:</b> TSAI, Li-Huei; 81 Farnham Street, Belmont, MA 02178 (US). PATRICK, Gentry, N.; 1 Woodman Street, No. 1, Jamaica Plain, MA 02130 (US). LEE, Ming, Sum  <b>(74) Agents:</b> BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).	<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 27 July 2000 (27.07.00)	
<b>(54) Title:</b> METHODS AND COMPOSITIONS FOR TREATING NEURODEGENERATIVE DISEASES  <b>(57) Abstract</b>  The present invention relates to methods of preventing or treating neurodegenerative diseases by administering an antagonist or inhibitor of p25. In particular, the invention relates to methods of preventing or treating a neurodegenerative disease by administering a calpain antagonist or inhibitor, or a cation antagonist or inhibitor, which reduces the truncation or conversion of p35 to p25. It also relates to p.35 and antibodies directed to p.25 and cdk 5. It relates to methods of inhibiting p25/cdk association, cdk 5 deregulation and phosphorylation of tan. Methods of assessing neurodegenerative diseases using p 25 are provided.		

(51) International Patent Classification<sup>7</sup>: (Continued)

A61P 25/28, 25/16, G01N 33/68, C07K 16/18, C12N 15/12 // (A61K 38/55, 38:17) (A61K 38/17, 38:05) (A61K 38/55, 31:00) (A61K 38/17, 31:00)

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 99/24221

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 7 A61K38/55 A61K38/05 A61K38/17 A61K31/00 A61P25/28 A61P25/16 G01N33/68 C07K16/18 C12N15/12 //(A61K38/55, 38:17),(A61K38/17,38:05),(A61K38/55,31:00),(A61K38/17,31:00) According to International Patent Classification (IPC) or to both national classification and IPC														
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 7 A61K C07K C12N G01N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practical, search terms used)														
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <table border="1"> <thead> <tr> <th>Category *</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>WANG KKW ET AL.: "CALPAIN INHIBITION: AN OVERVIEW OF ITS THERAPEUTIC POTENTIAL" TRENDS IN PHARMACOLOGICAL SCIENCES, vol. 15, no. 11, November 1994 (1994-11), pages 412-417, XP000568894 the whole document</td> <td>1,2,5-7, 16-20, 24-26, 28,29, 41,42</td> </tr> <tr> <td>Y</td> <td>_____</td> <td>31,32</td> </tr> <tr> <td>X</td> <td>EP 0 580 161 A (MCLEAN HOSPITAL CORP) 26 January 1994 (1994-01-26)  page 1 -page 5; claims  -/-</td> <td>1,2,5-7, 16-20, 24-26, 28,29, 41,42</td> </tr> </tbody> </table>			Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X	WANG KKW ET AL.: "CALPAIN INHIBITION: AN OVERVIEW OF ITS THERAPEUTIC POTENTIAL" TRENDS IN PHARMACOLOGICAL SCIENCES, vol. 15, no. 11, November 1994 (1994-11), pages 412-417, XP000568894 the whole document	1,2,5-7, 16-20, 24-26, 28,29, 41,42	Y	_____	31,32	X	EP 0 580 161 A (MCLEAN HOSPITAL CORP) 26 January 1994 (1994-01-26)  page 1 -page 5; claims  -/-	1,2,5-7, 16-20, 24-26, 28,29, 41,42
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	WANG KKW ET AL.: "CALPAIN INHIBITION: AN OVERVIEW OF ITS THERAPEUTIC POTENTIAL" TRENDS IN PHARMACOLOGICAL SCIENCES, vol. 15, no. 11, November 1994 (1994-11), pages 412-417, XP000568894 the whole document	1,2,5-7, 16-20, 24-26, 28,29, 41,42												
Y	_____	31,32												
X	EP 0 580 161 A (MCLEAN HOSPITAL CORP) 26 January 1994 (1994-01-26)  page 1 -page 5; claims  -/-	1,2,5-7, 16-20, 24-26, 28,29, 41,42												
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.														
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (see specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family														
Date of the actual completion of the international search  3 May 2000		Date of mailing of the international search report  17/05/2000												
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3018		Authorized officer  Teyssier, B												

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Initial Application No  
PCT/US 99/24221

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 11850 A (CORTEX PHARMA INC ;GEORGIA TECH RES INST (US)) 23 July 1992 (1992-07-23)  claims	1,2,5-7, 16-20, 24-26, 28,29, 41,42
X	WO 98 41092 A (SMITHKLINE BEECHAM CORP ;DAINES ROBERT A (US); SHAM KELVIN KIN CHE) 24 September 1998 (1998-09-24)  page 1	1,2,5-7, 16-18, 24,25, 28,29, 41,42
X	WO 98 25883 A (BASF AG ;MOELLER ACHIM (DE); LUBISCH WILFRIED (DE); TREIBER HANS J) 18 June 1998 (1998-06-18)  page 1 -page 3; claims 6,9	1,2,5-7, 16-18, 24,25, 28,29, 41,42
X	WO 98 12210 A (SYNPHAR LAB INC ;CANADA NAT RES COUNCIL (CA)) 26 March 1998 (1998-03-26)  page 1 -page 2	1,2,5-7, 16-18, 24,25, 28,29, 41,42
X	DATABASE WPI Section Ch, Week 199809 Derwent Publications Ltd., London, GB; Class B03, AN 1998-100810 XP002136734 & WO 98 01129 A (MITSUBISHI CHEM CORP), 15 January 1998 (1998-01-15) abstract	1,2,5-7, 16-18, 24,25, 28,29, 41,42
X	US 5 554 767 A (YUEN PO-WAI ET AL) 10 September 1996 (1996-09-10)  column 1; claim 8	1,2,5-7, 16-18, 24,25, 28,29, 41,42
X	WO 96 21655 A (HOECHST MARION ROUSSEL INC) 18 July 1996 (1996-07-18)  page 5, line 2 - line 13  -/-	1,2,5-7, 16-18, 24,25, 28,29, 41,42

# INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/US 99/24221

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 623 627 A (MITSUBISHI CHEM IND) 9 November 1994 (1994-11-09)  claim 12	1,2,5-7, 16-18, 24,25, 28,29, 41,42
X	WO 98 20736 A (SUO ZHIMING ;ARENDASH GARY W (US); MULLAN MICHAEL (US); CRAWFORD F) 22 May 1998 (1998-05-22)  claim 1	1,2,5-7, 16,17, 21,22, 24,25, 27-30, 41,42
X	WO 97 10210 A (SMITHKLINE BEECHAM PLC ;HARLING JOHN DAVID (GB); ORLEK BARRY SIDNE) 20 March 1997 (1997-03-20)  page 8, line 4 - line 14	1,2,5-7, 16,17, 21,22, 24,25, 27-30, 41,42
X	WO 96 21641 A (SMITHKLINE BEECHAM PLC ;ORLEK BARRY SIDNEY (GB); HARLING JOHN DAVI) 18 July 1996 (1996-07-18)  page 7, line 14 - line 24	1,2,5-7, 16,17, 21,22, 24,25, 27-30, 41,42
X	WO 95 33723 A (SMITHKLINE BEECHAM PLC ;BROWN THOMAS HENRY (GB); COPPER DAVID GWYN) 14 December 1995 (1995-12-14)  page 8, line 26 - line 34	1,2,5-7, 16,17, 21,22, 24,25, 27-30, 41,42
X	WO 95 11240 A (SMITHKLINE BEECHAM PLC ;COOPER DAVID GWYN (GB); KING RONALD JOSEPH) 27 April 1995 (1995-04-27)  page 10, line 8 - line 15	1,2,5-7, 16,17, 21,22, 24,25, 27-30, 41,42
A	US 5 051 403 A (MILJANICH GEORGE P ET AL) 24 September 1991 (1991-09-24) claims	23
X	WO 97 20842 A (CENTRE NAT RECH SCIENT ;MEIJER LAURENT (FR); BISAGNI EMILE (FR); L) 12 June 1997 (1997-06-12) page 9, line 8 - line 19; claim 18	8,13,42
	-/-	

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/24221

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 48394 A (FUELL DIANE ;SMITHKLINE BEECHAM PLC (GB)) 24 December 1997 (1997-12-24) the whole document	31, 32
X	LEE KI-YOUNG ET AL: "Interaction of cyclin-dependent kinase 5 (Cdk5) and neuronal Cdk5 activator in bovine brain." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 3, 19 January 1996 (1996-01-19), pages 1538-1543, XP002136729	42, 44-46
A	page 1542, column 1, line 5 - line 10 page 1540, column 1, line 25 - line 27 page 1542, column 2, line 11 -page 1543 the whole document	1-41
X	UCHIDA T ET AL: "Precursor of cdk5 activator, the 23 kDa subunit of tau protein kinase II: Its sequence and developmental change in brain." FEBS LETTERS, vol. 355, no. 1, 21 November 1994 (1994-11-21), pages 35-40, XP002136730	42, 43
A	page 36, paragraph 2.6 the whole document	1-41
A	IMAHORI K ET AL: "Physiology and pathology of tau protein kinases in relation to Alzheimer's disease." JOURNAL OF BIOCHEMISTRY, vol. 121, no. 2, February 1997 (1997-02), pages 179-188, XP002136731	
A	NAKAMURA S ET AL: "p35-nck5a and cyclin-dependent kinase 5 colocalize in Lewy bodies of brains with Parkinson's disease." ACTA NEUROPATHOLOGICA, vol. 94, no. 2, August 1997 (1997-08), pages 153-157, XP000906828	
T	PATRICK GENTRY N ET AL: "Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration." NATURE, vol. 402, no. 6762, 9 December 1999 (1999-12-09), pages 615-622, XP002136732 page 620, column 2	1-46
	-/-	

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)



# INTERNATIONAL SEARCH REPORT

Int. J. Application No.  
PCT/US 99/24221

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>MANDELKOW E: "Alzheimer 's disease. The tangled tale of tau." NATURE, vol. 402, no. 6762, 9 December 1999 (1999-12-09), pages 588-589, XP002136733 the whole document</p>	1-46

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 24221

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-9, 13, 14, 16-32 and 41 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged/effects of the compound/composition.
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependant claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 10-12, 15 (all completely); 1, 5-9, 13, 14, 16, 41, 42 (all in part)

1°/ Present claims 1, 5-9, 13, 14, 16, 41, 42 relate to compounds defined by reference to desirable properties, namely inhibition of conversion of p35 to p25, reduction of p25 accumulation, regulation of cdk5 kinase, inhibition of binding of p25 to cdk5. The claims cover all compounds having one of these properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been restricted to those compounds which appear to be clear, supported and disclosed, namely calpain inhibitors (as illustrated by the compounds recited in claims 19 and 26), calcium antagonists (as illustrated in claim 23 by omega conotoxin alone; references to MKA01 and Sb201823-A without mention of structure or bibliographic data to those code names are insufficient for clear identification of the claimed compounds), purine-based cdk inhibitors (as illustrated in the description by roscovitine and olomoucine), and antibodies to p25 or cdk5.

2°/ Present claims 10-12 and 15 attempt to link, by reference to claims 9 and 14, calpain inhibitors or calcium antagonists with regulation of cdk5 kinase or p25/cdk5 association while the description does not suggest any direct action of calpain or calcium on formation and/or activity of cdk5 complexes. Therefore those claims fail to meet the requirements of Article 6 PCT that claims are supported by the description. Consequently, those claims have not been searched.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No

PCT/US 99/24221

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0580161	A	26-01-1994	AT 173088 T	15-11-1998
			CA 2101117 A	23-01-1994
			DE 69321893 D	10-12-1998
			DE 69321893 T	12-05-1999
			EP 0582143 A	09-02-1994
			JP 7225231 A	22-08-1995
			US 5624807 A	29-04-1997
			US 5928885 A	27-07-1999
			CA 2101417 A	15-02-1994
			JP 6172211 A	21-06-1994
WO 9211850	A	23-07-1992	AU 5590596 A	22-08-1996
			AU 667463 B	28-03-1996
			AU 9152791 A	17-08-1992
			CA 2098609 A	29-06-1992
			EP 0564552 A	13-10-1993
			JP 6504061 T	12-05-1994
			US 5444042 A	22-08-1995
WO 9841092	A	24-09-1998	NONE	
WO 9825883	A	18-06-1998	AU 5752398 A	03-07-1998
			BG 103485 A	31-01-2000
			EP 0944582 A	29-09-1999
			HR 970680 A	31-10-1998
			NO 992821 A	11-06-1999
			PL 334059 A	31-01-2000
WO 9812210	A	26-03-1998	AU 4133597 A	14-04-1998
			EP 0929571 A	21-07-1999
			US 5916887 A	29-06-1999
WO 9801129	A	15-01-1998	JP 10101560 A	21-04-1998
US 5554767	A	10-09-1996	US 5760048 A	02-06-1998
WO 9621655	A	18-07-1996	AU 692044 B	28-05-1998
			AU 4473196 A	31-07-1996
			CA 2210258 A	18-07-1996
			CN 1173174 A	11-02-1998
			EP 0802909 A	29-10-1997
			FI 972935 A	10-07-1997
			HU 77649 A	28-07-1998
			JP 10512257 T	24-11-1998
			NO 973216 A	09-09-1997
			NZ 298999 A	28-01-1999
			US 5691368 A	25-11-1997
			ZA 9600100 A	24-07-1996
EP 0623627	A	09-11-1994	AT 145214 T	15-11-1996
			CA 2121523 A	29-10-1994
			DE 69400887 D	19-12-1996
			DE 69400887 T	22-05-1997
			DK 623627 T	28-04-1997
			ES 2096370 T	01-03-1997
			GR 3022524 T	31-05-1997
			JP 7048340 A	21-02-1995
			US 5506243 A	09-04-1996

Form PCT/ISA/210 (patent family annex) (July 1992)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/24221

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9820736 A	22-05-1998	US 6011019 A AU 5165498 A	04-01-2000 03-06-1998
WO 9710210 A	20-03-1997	EP 0859760 A	26-08-1998
WO 9621641 A	18-07-1996	AU 4537596 A EP 0802899 A JP 10512252 T US 5863952 A	31-07-1996 29-10-1997 24-11-1998 26-01-1999
WO 9533723 A	14-12-1995	EP 0763022 A JP 10500698 T	19-03-1997 20-01-1998
WO 9511240 A	27-04-1995	AU 7855394 A EP 0724578 A JP 9504014 T ZA 9408232 A	08-05-1995 07-08-1996 22-04-1997 22-04-1996
US 5051403 A	24-09-1991	AT 140388 T AU 648052 B AU 6964091 A CA 2045473 A DE 69027865 D DE 69027865 T DK 593450 T EP 0593450 A ES 2091906 T GR 3021087 T HK 1006810 A JP 5501715 T WO 9107980 A US 5424218 A US 5189020 A US 5559095 A US 5264371 A	15-08-1996 14-04-1994 26-06-1991 23-05-1991 22-08-1996 02-01-1997 25-11-1996 27-04-1994 16-11-1996 31-12-1996 19-03-1999 02-04-1993 13-06-1991 13-06-1995 23-02-1993 24-09-1996 23-11-1993
WO 9720842 A	12-06-1997	FR 2741881 A CA 2238843 A EP 0874847 A JP 2000501408 T	06-06-1997 12-06-1997 04-11-1998 08-02-2000
WO 9748394 A	24-12-1997	EP 0914117 A	12-05-1999

Form PCT/ISA/210 (patent family annex) (July 1992)